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The Human ARF Tumor Suppressor Regulates Drosha Nucleolar Localization and rRNA Processing Activity

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by
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

APC1:	Anaphase-Promoting Complex Subunit 1
ARF:	Alternate Reading Frame
ATM:	Ataxia Telangiectasia Mutation
ATP:	Adenosine Triphosphate
ATR:	ATM and RAD3-Related
BH3:	Bcl-2 Homology 3
BRCA1:	Breast Cancer Susceptibility Gene 1
CDK:	Cyclin Dependent Kinase
CDK2:	Cyclin Dependent Kinase 2
CDK4:	Cyclin Dependent Kinase 4
CDKN1:	Cyclin Dependent Kinase Inhibitor 1
CDKN2:	Cyclin Dependent Kinase Inhibitor 2
cDNA:	Complementary DNA
CHK2:	Checkpoint Kinase 2
CIN:	Chromosomal Instability
CRISPR:	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI:	4',6-Diamidino-2-Phenylinodle
DDR:	DNA Damage Response
DDX17:	DEAD Box Protein 17
DDX3:	DEAD Box Protein 3

DDX5:	DEAD Box Protein 5
DGCR8:	Digeorge Syndrome Critical Region 8
DHX33:	DEAH-Box Helicase
DKC1:	Dyskeratosis Congenita Protein 1
dsRNA:	Double Stranded Ribonucleic Acid
EDTA:	Ethylene Diamine Tetraacetic Acid
EGFR:	Epidermal Growth Factor Receptor
eIF:	Eukaryotic Initiation Factor
EMT:	Epithelial Mesenchymal Transition
GIN:	Genomic Instability
AgNOR:	Silver-Stained Nucleolar Organizing Region
GTP:	Guanosine Triphosphate
HDAC:	Histone Deacetylase
HDM2:	Human Double Minute 2
HMEC:	Human Mammary Epithelial Cell
hnRNPM4:	Heterogeneous Nuclear Ribonucleoprotein M
HSP70:	Heat Shock Protein 70
hTERT:	Human Telomerase Reverse Transcriptase
INK4:	Inhibitor of Cyclin-Dependent Kinase 4
IRES:	Internal Ribosome Entry Site
siRNA:	Small Interfering Ribonucleic Acid
ISG15:	Interferon Stimulated Gene 15 Kda
MAPK:	Mitogen-Activated Protein Kinase

MDC1:	Mediator of DNA Damage Checkpoint 1
MDM2:	Mouse Double Minute 2
miRNA:	Micro Ribonucleic Acid
MMP11:	Matrix Metalloproteinase 11
MMP7:	Matrix Metalloproteinase 7
mRNA:	Messenger Ribonucleic Acid
mRNP:	Messenger Ribonucleoprotein
mTOR:	Mammalian Target of Rapamycin
mTORC1:	Mammalian Target of Rapamycin Complex 1
MTS1:	Metastasis Associated Protein
NMD:	Nonsense-Mediated Decay
NOR:	Nucleolar Organizing Region
NPM/NPM1:	Nucleophosmin
ORF:	Open Reading Frame
PABP:	Poly(A)-Binding Protein
Pol I:	Ribonucleic Acid Polymerase I
Pol II:	Ribonucleic Acid Polymerase II
Pol III:	Ribonucleic Acid Polymerase III
PRC1:	Protein Regulator of Cytokine 1
PUMA:	P53-Upregulated Modulator of Apoptosis
RB:	Retinoblastoma Protein
rDNA	Ribosomal Deoxyribonucleic Acid
RISC:	RNA-Induced Silencing Complex

RNA:	Ribonucleic Acid
RP:	Ribosomal Protein
RPL11:	Ribosomal Protein, Large 11
RPL5:	Ribosomal Protein, Large 5
rRNA:	Ribosomal Ribonucleic Acid
shRNA:	Short Hairpin Ribonucleic Acid
siRNA:	Small Interfering Ribonucleic Acid
smARF:	Small ARF
snoRNP:	Small Nucleolar Ribonucleoprotein
SUMO:	Small Ubiquitin-Like Modifier
TBX2:	T-Box Protein 2
TBX3:	T-Box Protein 3
TGF:	Transforming Growth Factor
TLHMEC:	Large T and hTERT - Human Mammary Epithelial Cells
tRNA:	Transfer Ribonucleic Acid
$tRNA^{MET}$:	Methionine –Transfer RNA
TRRAP:	Transformation/Transcription Domain-Associated Protein
TTF1:	Transcription Termination Factor, RNA Polymerase I
UBF1:	Upstream Binding Factor 1
USP7:	Ubiquitin-Specific Processing Protease 7
UTR:	Untranslated Region
UV:	Ultra Violet

VEGF:	Vascular Endothelial Growth Factor
WT:	Wild Type
XIAP:	X-Linked Inhibitor of Apoptosis

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Dedicated to my parents

ABSTRACT OF THE DISSERTATION

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Ribosomes are vital to the survival of a cell, as they are directly responsible for the synthesis of proteins, which perform critical cellular functions. As such, majority of the energy reserves in a proliferating cell are expended towards synthesis of ribosomes. Cancer cells, with their enhanced proliferation rates, tend to upregulate ribosome biogenesis in order to meet the demand for increased protein synthesis necessary to sustain rapid proliferation. Many of the oncogenes and tumor suppressors known to be deregulated in cancers are capable of positively and negatively regulating ribosome biogenesis, respectively. The ARF tumor suppressor strongly suppresses ribosome biogenesis, particularly in presence of oncogenic signaling. Furthermore, ARF is capable of negatively regulating multiple oncogenes capable of driving tumorigenesis partly through the ribosome biogenesis pathway. As ARF loss is a frequent occurrence in cancer cells, delineating the ARF-regulatory network and determining the impact of ARF loss on this network can give significant insight into the biology of ARF-deficient tumor cells.

Expression of the RNase III enzyme, Drosha, has been reported to have prognostic value in multiple cancers. However, Drosha expression appears to have a dual nature in tumorigenesis, as both overexpression and loss of Drosha have been reported to have tumorigenic functions. Although the mechanistic basis of this apparent duality are not yet known, gaining a deeper understanding of Drosha's functional capabilities can give us an insight into its role in tumorigenesis. Drosha performs critical functions in biogenesis of multiple RNA species within the cell, including ribosomal RNA (rRNA), micro RNA (miRNA) and messenger RNA (mRNA). Drosha's role in miRNA biogenesis is the most studied and characterized aspect of its functions and can explain the tumor suppressive aspect of its dual nature; a global decrease in miRNAs has been reported to be part of tumor progression, and loss of Drosha has the potential to significantly deplete mature miRNA population within the cell. However, how overexpression of Drosha can drive tumorigenesis remains to be studied. As enhanced ribosome biogenesis is another feature of cancer cells and Drosha has been shown to aid in processing of r RNA, Drosha's role in ribosome biogenesis pathway has the potential to function in an oncogenic manner. Therefore, further characterization of Drosha's role in ribosome biogenesis can significantly enhance our understanding of its contribution to tumorigenesis.

Recent studies in mouse cell lines revealed that ARF tumor suppressor is capable of negatively regulating Drosha expression in a translation-dependent manner. Given the entrenched role of ARF in inhibiting ribosome biogenesis, I hypothesized that ARF's ability to regulate Drosha could impact Drosha's functions in ribosome biogenesis pathway. I further hypothesized that Drosha overexpression could function in a pro-proliferative manner through the ribosome biogenesis pathway. The data presented in this Dissertation reveals that human p14ARF is capable of regulating Drosha protein expression in a dynamic and localized fashion; loss of ARF increases

over all cellular Drosha protein levels and also the localization of Drosha to the nucleolus. ARF potentially regulates nucleolar localization of Drosha by sequestering it away from nucleolus, as we found that ARF immunoprecipitated with Drosha in RNA-independent manner. Furthermore, loss of ARF enhances ribosome biogenesis both at the level of 47s rRNA transcription and processing. Association of Drosha with precursor rRNAs was also enhanced in absence of ARF, suggesting that enhanced nucleolar localization of Drosha upon ARF loss contributes to rRNA processing. Drosha overexpression by itself was able to increase ribosome biogenesis, with a modest increase in 47s rRNA transcription and a faster accumulation of 28s and 18s rRNAs. Drosha overexpression led to an increase in ARF expression, although this induction of ARF was not sufficient to inhibit Drosha's ability to enhance ribosome biogenesis and cell proliferation. However, overexpression of ARF negated proliferative enhancement induced by Drosha overexpression. These results point towards a cross-regulatory loop between ARF and Drosha, with functional impact on ribosome biogenesis.

EPIGRAPH

“It is the mark of an educated mind to be able to entertain a thought without accepting it.”

~Aristotle

CHAPTER 1:

INTRODUCTION & SIGNIFICANCE

1.1 Cancer: A Therapeutic Challenge

Cancer treatment has been a challenge faced by physicians for millennia, as evidenced by the documentation of cancer diagnosis and its treatment in one of the oldest surviving surgical document from Ancient Egypt (3000-1600 B.C.), the Edmund Smith Surgical Papyrus (nih.gov, URL: https://ceb.nlm.nih.gov/proj/ttp/flash/smith/smith.html). According to this document, apart from a form of surgical intervention in which the tumor mass may get destroyed through cauterization, tumors in general were considered to have no treatment (cancer.org, URL: http://www.cancer.org/cancer/cancerbasics/thehistoryofcancer/the-history-of-cancer-what-is-cancer). Since then, thanks to a significant amount of research and study that went into understanding tumor biology, we have gained tremendous insight into the molecular mechanisms that lead to tumor formation and progression. Between early screening procedures, surgery, radiation therapy, chemotherapy, and in some cases, vaccines, we have come a long way in our ability to treat cancers and improve life expectancy of cancer patients. Yet, based on the statistics provided by the World Cancer Report 2014, cancer is amongst one of the leading causes of death worldwide, with a diagnosis of approximately 14 million new cancer cases and 8.2 million cancer-related deaths in 2012 (World Health Organization, URL: http://www.who.int/mediacentre/factsheets/fs297/en/). Within United States, the American Cancer Society predicts approximately 1.7 million new cancer cases and 600,000 cancer-related deaths in 2016 (cancer.org, URL: http://www.cancer.org/acs/groups/content/@research/documents/document/acspc-047079.pdf). These statistics dictate the need for continued cancer research and development of novel therapeutic interventions.

Tumors can present different histopathologic properties, with early stage tumors having a tendency to be restricted to the sites of origin and later stage tumors having more invasive properties, where tumor cells invade into the surrounding tissue and subsequently metastasize to distal organs and tissues. For a long time, surgical resection has been the predominant form of intervention in cancer treatment. However, while surgical intervention during early stages of tumorigenesis has a better disease-free-survival rates, it has little effect in managing later stage invasive and metastatic disease states (1). Early detection of cancer therefore is of high importance in disease management. In early 1900s, a novel radiation-based therapy—brachytherapy—in which radioactive seeds were inserted directly into tumor mass, had great success in improving survival rates of gynecological cancer patients (2,3). Concomitant with development of new radioactive isotopes and techniques for radiation delivery, adjuvant radiation therapy became more mainstream for cancer treatment (4-9) and continues to be so to this day (10-14). While these adjuvant therapies have better survival statistics for early stage cancers, invasive and metastatic cancers still pose a challenge for therapy.

In addition to radiation therapy, 1900s also saw development of chemotherapeutic agents in cancer treatment. In 1947, Sidney Farber, considered to be the father of modern chemotherapy, reported the first chemotherapeutic intervention in treating pediatric leukemia through intramuscular injection of aminopterin, a synthetic antagonist of folic acid (15). Although the remission observed with this treatment lasted for only three months and also had toxic side effects, it paved the way for discover of new drugs for cancer treatment. Since then, over 200 chemotherapeutic drugs have been discovered to date, for the purpose of cancer treatment (cancer.org, URL: https://www.cancer.gov/about-cancer/treatment/drugs). Many of these drugs are designed to target a specific protein, or a specific family of proteins, in order to achieve

cytotoxicity in their target cells. In addition to a challenge in targeting these molecules specifically to cancer cells without effecting normal cells, drug treatment has also lead to the emergence of drug-resistant clones, causing cancer remission (16-19). Therefore, an improved method for cancer cell-specific delivery of therapeutic drugs as well as a mechanistic understanding of how drug resistance arises in cancer cells are necessary for the development of successful therapeutic strategy.

Cancer arises due to an uncontrolled cell growth and proliferation, and can lead to disruption of normal function of the tissue or organ in which it arises. The basis of this uncontrolled cell proliferation is alterations in the genetic material, or the DNA, that lead to abnormal functioning of proteins, which establish the pattern for cell behavior. Cancer, therefore, is a genetic disease, in many cases, with an inherent genomic instability leading to a constantly evolving genetic makeup (20-26) that can give rise to a heterogeneous population of cells within a tumor mass. Both the genomic instability and the tumor heterogeneity can significantly deter chemotherapeutic intervention, the former by enabling emergence of genetic contexts in which the drug target becomes obsolete while the latter allows for outgrowth of clones that function independent of the drug target. Understanding the genetic nature of tumor cells that determine cancer's behavior can therefore significantly help overcome the current challenges in therapeutic intervention.

1.2 Jumping Through Hoops: An Obstacle Course to Neoplastic Transformation

The genetic context that gives rise to cancer is, at its roots, a combination of two events: upregulation of genes that enable indefinite proliferation (oncogenes) and downregulation of genes

that prohibit proliferation (tumor suppressors). Both of these will be further discussed below in the context of protective barriers that normal cells need to overcome in order to become neoplastic.

Cell cycle checkpoints

Normal cell proliferation involves passage of cells through distinct phases of cell cycle, during which cells amass the necessary nutrients required for successful cell division, replicate their DNA, and faithfully segregate the DNA and cytoplasmic contents between daughter cells. The cell cycle includes several phases: 1) the G1 phase, in which cells accumulate the proteins and enzymes necessary for DNA replication; 2) the S phase, in which cells replicate their DNA; 3) the G2 phase, in which the cells ensure successful completion of DNA replication and prepare for mitotic cell division; and 4) the M phase, in which the replicated copies of DNA (sister chromatids) are separated and divided between two daughter cells during cytokinesis (cell division) (27). The cell cycle is a tightly regulated process, aberrations in which can lead to cell cycle arrest at one of three checkpoints: 1) the G1-S checkpoint, which prevents entry into the S phase in conditions of insufficient nutrient availability, DNA damage, or inappropriate cell division in previous cycle (28), 2) the G2-M checkpoint, which prevents entry into mitosis in conditions of DNA damage (29,30) and 3) spindle checkpoint, which prevents cell cycle progression in cases of improper mitotic spindle assembly (31). The purpose of all of these checkpoints is maintenance of genomic integrity and equivalent distribution of complete genome between dividing cells.

Cell proliferation is triggered in response to mitogenic signaling, in absence of which cells are maintained in a quiescent G0 state. Binding of growth factors to growth factor receptors in a cell membrane leads to signaling cascades that result in upregulation of transcription factors, which regulate expression of genes necessary for cell cycle entry and progression (32-34). The cell cycle

progression depends on the activity of different cyclin and cyclin-dependent kinase (CDK) protein complexes, with the activity of a different complex controlling entry into different phases of the cell cycle (35). Activity of these complexes can be regulated through protein expression (expression of cyclins is transcriptionally regulated to increase at the appropriate phase of the cell cycle) as well as protein modification (activity of CDKs is regulated in a phosphorylation-dependent manner). Upregulation of pro-proliferative oncogenes, from growth factor receptors (EGFR) to transcription factors (Myc and E2F1) to cyclins (Cyclin D1 and Cyclin E) themselves, is a common feature of cancer cells (36-40). However, oncogene expression leads to activation of DNA damage response (DDR), which induces cell cycle arrest through the Rb and p53 cell cycle checkpoint pathways (41). The Rb tumor suppressor pathway predominantly controls entry of cells into the S phase, with Rb suppressing transcriptional activity of E2F transcription factors, which transcribe the S phase-related genes (42,43). The p53 tumor suppressor is capable of inducing both G1 and G2 checkpoint through its transcriptional regulation of the CDK-inhibitor, p21 (44). While cell cycle arrest through checkpoint activation can be transitory, with cells sometimes reentering the cycle after DNA repair and DDR signaling deactivation, oncogene expression appears to induce a permanent cell cycle arrest (senescence) (45-50). Senescence onset is dependent on accumulation of p21 and p16 CDK inhibitors, and functions through the p53 and Rb pathways (51-53). Deactivation of cell cycle checkpoints and the senescence pathway is critical for achieving unobstructed cell proliferation. As such, deactivation of p53 and Rb pathways is a common occurrence in cancer cells (54-57).

Cell Death: wrecker of the best-laid plans

While hyper-activation of pro-proliferative signals and deactivation of checkpoints can allow cells to proliferate without restriction, oncogene expression is also capable of activating programed cell death (apoptosis) (58,59). Increased incidence of apoptosis is frequently seen in pre-cancerous lesions potentially functioning as an anti-cancer barrier (60). Therefore, deactivation of this pathway is critical for cancer cell survival. The apoptotic cascade mainly functions through two different pathways – the intrinsic and the extrinsic apoptotic pathways (61). Internal cellular stresses such as DNA damage activate the intrinsic pathway, while activation of the extrinsic pathway depends on death signals from outside the cell binding to the tumor necrosis factor (TNF) death receptor family of proteins on the cell surface. Both pathways lead to activation of a set of caspases, which are cysteine-aspartic acid proteases with the ability to cleave and activate several cellular proteins involved in the apoptotic cascade, culminating in fragmentation and condensation of DNA. Activating mutations in anti-apoptotic genes (Eg. Bcl-xL, Bcl-2, and Mcl-1) and deactivating mutations in pro-apoptotic genes (Eg. Bax, and Bak) have been reported to be an integral part of tumor progression (62-64). Particularly, the p53 pathway has been shown to play a critical role in inducing the apoptotic pathway in response to several cellular stresses (65-68). As mentioned before, deregulation of p53 pathway occurs at high frequency in cancer cells and has the dual function of negating the cell cycle checkpoint as well as the apoptotic pathways.

Telomere erosion and contact inhibition

Unrestricted proliferation in addition to evasion of cell death can be highly advantageous in neoplastic transformation. However, a few more defense mechanisms such as telomere erosion and contact inhibition can further undermine proliferation of aberrant cells. Telomeres are the

protective ends on chromosomes which prevent chromosome end-to-end fusion thereby protecting genomic integrity (69). Under normal conditions, telomere length has a tendency to get shorter with progressive DNA replication cycles, eventually leading to chromosome fusions and formation of di-centric chromosomes whose resolution during mitotic division can cause chromosome breakage and genetic imbalances between daughter cells. Normally, cells tend to prevent proliferation of such cells through cell cycle arrest or apoptosis. However, in cells with aberrant checkpoint or apoptotic pathways, telomere erosion and subsequent genomic alterations can lead to genomic instability, with massive gains and losses in genetic material with each cell division (70). While genomic instability is a hallmark of cancers and has been proposed to drive tumor progression and contribute to tumor heterogeneity and therapeutic resistance (20,22,24,25), genetic instability can also be detrimental to cell viability. Therefore, as a means to prevent excessive chromosome breakage events and loss of crucial genetic material, cancer cells tend to activate expression of telomerase, a ribonucleoprotein with the ability to add telomere repeats at DNA ends as a protective measure (71).

Contact-inhibition of proliferation is another barrier for indefinite proliferation and limits proliferation of cells to a single monolayer. Mechanistically, cell-cell adhesion molecules (such as E-cadherin) have been shown to play a critical role in inhibiting proliferation when cells come in contact with each other (72,73). E-cadherin, with the aid of β-catenin and α-catenin, is capable of activating the HIPPO pathway, which can inhibit transcription of pro-proliferative genes such as Myc (74). Therefore, cell-cell contact can significantly impair the ability of aberrant cells to proliferate indefinitely. Hence, deactivation of the E-cadherin mediated inhibition of cell proliferation is another critical event for tumor progression. Indeed, loss of E-cadherin expression

is a major contributor to epithelial-mesenchymal transition (EMT), a phenomenon that increases the metastatic potential of tumor cells (75-77).

Essentially, the path to tumorigenesis involves deactivation and deregulation of several critical cellular pathways. While the aberrations in the above mentioned pathways can heavily contribute to tumorigenesis, several other pathways, such as angiogenesis and immune evasion, also play important roles in tumor progression and malignancy (25). Tumors, therefore, arise through a combined action of multiple "deregulated" pathways. Unfortunately, despite our mechanistic understanding of many of the pathways involved in tumorigenesis, development of a so called "cure" for cancer still remains elusive. The main reason for this is tumor heterogeneity, which arises from the varying genetic makeup of cells present within a tumor mass, and contributes to the development of drug resistance and tumor remission (78-80). Therefore, methodology that can identify heterogeneous mutational status of individual tumors can go a long way in helping us tackle the drug resistance problem.

1.3 Therapeutic Strategies Revisited

Advent of whole genome sequencing – in particular, cancer genome sequencing — has enabled us to identify the genetic alterations present within individual tumors (81-90). Thanks to decades of research, we have a fair amount of insight into cellular pathways that regulate normal cell behavior and how perturbations in these pathways contribute to tumorigenesis. With the huge amount of data available to us from cancer genome sequencing studies, the challenge we now face is in determining the "driver" mutations, as well as the permutations and combinations of these mutations, that enable proliferation and survival of aberrant cells. This can help in the development

of combination therapies that can preemptively target multiple "driver" pathways at once and hopefully diminish emergence of resistant clones.

Alternate to targeting therapy to specific signaling pathways, development of therapeutic methods targeting general features of cancer cells are also being considered. As an example, chromosomal instability, as has been mentioned before, is a hallmark of cancer cells and excessive instability can be detrimental to cells, normal or cancers. Therefore, several strategies are being used to induce catastrophic genomic instability in cancer cells through DNA damaging agents, to induce cell death (91).

Another example is the targeting of ribosome biogenesis, the pathway which gives rise to ribosomes, the cellular protein translation machinery. As proteins are the ultimate determinants of cell behavior, perturbations that impede protein synthesis as a whole can be detrimental for cell survival. As the work presented in this thesis focuses on pathways regulating ribosome biogenesis, the next few sections will discuss ribosome biogenesis in further detail and outline current attempts in targeting this pathway for cancer therapy.

1.4 Ribosome Biogenesis: Synthesis of Translation-Efficient Ribosomes

The mammalian ribosome is composed of the large 60s and small 40s subunits containing four ribosomal RNA (rRNA) transcripts and several ribosomal proteins (RPs). Generation of translation-efficient ribosomes is a tightly regulated process with multiple steps that occur in the nucleolus, nucleus and cytoplasm. It needs the combined effort of all three RNA polymerases, where the RNA Polymerase I (Pol I) transcribes the 18s, 5.8s and the 28s rRNAs; RNA Polymerase II (Pol II) transcribes the RPs and non-ribosomal factors that function in modifying, folding and assembling the subunits; and the RNA Polymerase III (Pol III) transcribes the 5s rRNA. The 18s,

5.8s and 28s rRNAs are transcribed as a single 47s pre-rRNA transcript in the nucleolus, the sub-nuclear structure consisting of ribosomal DNA (rDNA) repeats that encode the 47s pre-rRNA, while the 5s rRNA is transcribed in the nucleus and transported into nucleolus (92,93). Adapter molecules including RNA helicases, ribonucleases and small nucleolar RNA-protein complexes (snoRNPs) associate with the 47s transcripts co-transcriptionally and aid in folding, processing, modifying and assembling the rRNAs and their associated RPs into pre-60s and pre-40s subunits (94). The pre-40s (made up of the 18s rRNA and small subunit RPs) and the pre-60s (made up of the 28s, 5.8s and 5s rRNAs along with large subunit RPs) are then exported to the cytoplasm where they undergo further maturation steps to become translation competent.

1.5 Translational Control: Ribosome-Dependent and –independent Mechanisms

Translation of mRNAs involves three steps: initiation, elongation and termination. In brief, translation initiation involves the following steps: 1) the GTP-bound eukaryotic initiation factor (eIF) 2 binds the initiator Methionine-transferRNA ($tRNA^{MET}$) and complexes with the 40s subunit along with a few other eIF proteins, making up the 43s preinitiation complex; 2) eIF4F complex (containing eIF4E, eIF4A and eIF4G) and eIF4B unwind the 5'CAP proximal region of mRNA by means of ATP hydrolysis, allowing binding of the 43s complex to the unwound region; 3) the 43s complex scans the 5' untranslated region (5'UTR) in a 5' to 3' direction for the initiation codon; and 4) the $tRNA^{MET}$ binds to start codon, leading to the release of initiation factors, allowing binding of the 60s subunit and formation of elongation competent 80s ribosomal complex (95). Studies done in reticulocytes as early as 1960s indicated that the 80s ribosomal complex (or monosome) was mostly inactive and active translation of mRNAs occurred on polysome structures,

where multiple ribosomes associate with a single mRNA transcript and generate multiple copies of protein in tandem (96,97). However, a monumental discovery made in *Saccharomyces Cerevisiae* earlier this year revealed that the 80s monosome is competent in translation and that the choice of mRNA translation on polysomes versus a monosome is used as a regulatory mechanism for controlling gene expression (98); it was shown that mRNAs with short open reading frames (ORFs), long and highly structured 5' UTRs, and short half-life were preferentially translated on monosomes as opposed to polysomes.

Structural elements present within the untranslated regions of mRNAs have been known to have great impact on translational efficiency of said mRNA (99). MicroRNA-mediated suppression of mRNA translation through regulatory sequences within the 3'UTR is a classic example of 3' UTR-mediated gene silencing (100). Furthermore, the 5' UTR sequences containing multiple initiation codons upstream of the actual start site can decrease translation efficiency due to initiation events from wrong start codons. Additionally, highly stable secondary structures within the 5'UTR can impede ribosome scanning for the start codon and thereby decrease translational efficiency in absence of adaptor proteins that can resolve these secondary structures (101,102). mRNAs encoding pro-growth and proliferation genes such as cyclin D and Myc have been shown to have highly structured 5'UTRs, and their translation efficiency has been linked to the mTORC1/S6K1 signaling cascade that can enhance RNA helicase activity of translation initiation factors (103). These mechanisms stress the importance of translation initiation factors in regulating translational efficiency of select mRNAs. It has further been reported that translation of mRNAs containing 5' terminal oligopyrimidine tract (TOP), while enhanced in presence of mitogenic signaling, did not require the S6K1 kinase activity (104). TOP mRNA encoding proteins involved in translation, such as ribosomal proteins, translation elongation factors and poly A

binding prtein (PABP), associate with polyribosomes in presence of mitogen signaling and dissociate from polysomes upon loss of the mitogenic stimuli. As mitogenic signals tend to increase ribosome biogenesis, whether a global increase in available ribosomes could contribute to this enhanced association of TOP mRNAs with polysomes has not been tested.

For a long time, ribosomes were thought to be passive translational machinery while extrinsic factors, such as the structural and sequential elements of mRNA UTRs and translation initiation factors, functioned as regulatory components for translation. However, several groups reported existence of "specialized ribosomes" that achieved selectivity in mRNA translation based on the RP composition of the ribosome as well as modifications present within the rRNA components of the ribosome (105,106). To give a few examples, mutations in DKC1 gene, which encodes for a pseudouridine synthase with functional significance in rRNA modification, causes abnormalities in skin and bone as well as predisposition to cancer. Analysis of mice with mutated *Dkc1* revealed an impairment in IRES (internal ribosome entry site)-dependent mRNA translation, suggesting an important role for DKC1 and RNA modifications in translation of IRES containing mRNAs (107). It was further reported that IRES-driven translation of p53 and p27 tumor suppressors as well as Bcl-xL and XIAP apoptotic factors were significantly impaired in DKC1 deficient cells (108-110). Surprisingly, this suppression of IRES-driven mRNA translation in DKC1 deficient cells does not appear to be a global event, as IRES-driven translation of VEGF mRNA was reported to increase upon DKC1 depletion (111). These results are indicative of complex mechanisms governing translational regulation. Whether the selectivity observed in this translational regulation is dependent on ribosomal composition, or on the IRES element remains to be tested. Regardless, the fact that rRNA modifications can alter ribosomal affinity to certain RNA structural elements suggests a more active role for the ribosome in translational regulation.

In addition to the rRNA modifications, the RP composition of ribosomes has also been shown to contribute to translational regulation. The ribosomal protein L26 was shown to bind the 5'UTR region of p53 and upregulating its translation in response to DNA damage (112). Furthermore, ribosomopathies are a group of diseases that arise due to defects in ribosome biogenesis genes. However, mutations in different ribosomal proteins show highly variable phenotypes and affect different tissue types based on the protein mutated. While these variations may be attributed to non-translational roles of the ribosomal proteins, mutations that effect rRNA transcription (Pol I mutations) and ribosome subunit assembly also generate varying phenotypes affecting specific tissue functions rather than whole organism (113). These results indicate that both the composition and functions of ribosomes may vary between different tissues, advocating a regulatory role for ribosomes in gene expression.

1.6 Nucleolar Stress Surveillance: A Non-Translational Role for Ribosomal Components

While the translational regulation exerted by ribosomes is rather impressive, the ribosome biogenesis pathway has been shown to have functional importance in a broader cellular context. In fact, components of the ribosome biogenesis pathway, from rDNA to ribosomal proteins, have been shown to play a significant role in cellular stress response. The ability of DNA damage to induce the p53 tumor suppressor pathway is very well studied. However, a hallmark study performed by Rubbi and Milner showed that cells were able to sustain high levels of DNA damage without activating p53 so long as the nucleolar functions remained intact, but disruption of nucleolus even in absence of DNA damage was able to stabilize p53 (114). To further strengthen these results, several of the chemotherapeutic drugs used for cancer treatment, presumably through

induction of DNA damage, were shown to have inhibitory functions in ribosome biogenesis (115). Mechanistically, it has been reported that the RPL5/RPL11/5s rRNA preribosomal complex is capable of stabilizing p53 by inhibiting MDM2-mediated suppression of p53 (116). Impairment of ribosome biogenesis can divert the 5s rRNA preribosomal complex from assembly into the 60s pre-ribosomal subunit to binding MDM2 and lifting its repression over p53. To further stress the p53-activating roles of ribosomal components, it was shown that the L11 protein was expressed at significantly higher levels in non-ribosomal fractions in splenocytes collected from Eμ-*Myc* transgenic mice that constitutively express c-Myc in B-cell lineage and are predisposed to B cell lymphomas at an early age. This L11 protein immunoprecipitates with MDM2 and is able to induce p53 expression in response to Myc overexpression, a potential tumor suppressive mechanism. However, nucleolar disruption is no longer able to activate p53 in cells containing mutant MDM2 incapable of binding L11 protein. As a proof of tumor suppressive functions of the L11-MDM2-p53 axis, MDM2 mutation in the Eμ-*Myc* background lead to significant acceleration of Myc induced lymphomagenesis. In addition to a p53-dependent suppression of Myc's oncogenic transformation, L11 is also capable of directly binding Myc and prevents recruitment of its transcriptional coactivator, TRRAP, thereby repressing its transcriptional potential (117).

The L11-MDM2 axis, in addition to its regulation of Myc-driven tumorigenesis, is also capable of inhibiting expression of the E2F-1 protein; MDM2 is capable of binding and stabilizing E2F-1 by preventing its ubiquitination and proteasomal degradation by the E3 ligase SCF^{SKP2} (118). Selective inhibition of Pol I transcription without altering Pol II and Pol III transcription lead to L11-medaited inhibition of MDM2, which caused a destabilization of E2F-1 protein and a subsequent cell cycle arrest in a p53-independent and RB-dependent manner (119). These results suggest that cellular perturbations that alter the stoichiometry of rRNA to ribosomal proteins

necessary for ribosome biogenesis can liberate rRNA and RPs from ribosomal fractions, allowing them to function in suppressing cell cycle progression.

To further link the nucleolus with global cellular responses, many of the proteins involved in regulating ribosome biogenesis also function in regulating cell cycle, DNA damage repair, DNA replication and even chromosome segregation during mitosis. To give a few examples, the ARF tumor suppressor has a significantly inhibitory role in ribosome biogenesis and is capable of inducing p53-dependent cell cycle checkpoint through inhibition of MDM2 in response to DNA damage (120,121). Both Nucleolin and Nucleophosmin (NPM), which have critical functions at multiple steps of ribosome biogenesis, have been shown to accumulate at sites of DNA double stranded breaks and to facilitate DNA repair (122-125). Furthermore, both proteins play critical functions in maintaining nuclear structure and potentially chromosome segregation, as depletion of either protein leads to formation of aberrant nuclear structures, such as micronuclei, and aberrant mitotic spindle (126-128). All these results point toward an intricate intermingling of nucleolar functions with global cellular processes. Several studies conducted on the nucleolar proteome give further strength to this idea; proteins kinases involved in cell cycle regulation (CDK2 and Aurora B), DNA damage response proteins (ATM), ribosome biogenesis regulatory proteins (p14ARF), and proteins involved in a wide variety of cellular processes have been found to be part of the nucleolar proteome and exhibit a dynamic redistribution in response to DNA damage (129-131).

The long reaching impact of perturbations in nucleolar components and functions, as evidenced by the literature reviewed above, stresses the importance of this sub-nuclear structure in overall cellular homeostasis. The extent of regulation that goes into inhibition of cell proliferation under conditions of nucleolar stress further emphasizes the importance of maintaining normal nucleolar functions in preventing abnormal cell behavior.

1.7 Ribosome biogenesis in cancer

Electron microscopic studies of the nucleolus identified three regions within the nucleolus: the fibrillar center, the dense fibrillar component and the granular component. The fibrillar center consists of the actively transcribing rDNA genes and transcription factors, the dense fibrillar component consists of the protein complexes involved in rRNA processing and the granular component contains the pre-ribosomal complexes (132,133). The rDNA sequences are considered to be the nucleolar organizing regions (NORs), as they recruit the rRNA transcription and processing machinery to their sites and promote formation of the nucleolar structures around them. The proteins associated with the NORs have a strong affinity for silver, enabling visualization of nucleoli through silver-staining methods (AgNOR staining). Characterization of the nucleolar structure and number in several cancer cells using different staining methods draw a strong correlation between the nucleolar area and proliferation rate – greater the nucleolar area, higher the rate of proliferation (134,135). Furthermore, nucleolar number and size have been shown to have prognostic value in multiple cancers, with increased nucleolar number and size associating with higher grade tumors and poorer prognosis (133,136-138). However, increased ribosome biogenesis (or nucleolar area), while found in may cancer cells, is not a characteristic shared by all cancer cells; some cancers have been shown to have similar or comparatively lower ribosome biogenesis rates than those observed in normal tissue. Nonetheless, enhanced ribosome biogenesis appears to promote tumorigenesis and tumor progression. Atypical endometrial lesions, which have been known to give rise to ovarian cancer, have upregulated expression of nucleolar RNA and ribosomal proteins, which persisted in ovarian cancers (139). Increased levels of ribosome biogenesis in hepatocytes from chronic liver disease were also shown to be associate with an

increased risk of developing hepatocellular carcinoma (140). To further emphasis pro-tumorigenic contributions of ribosome biogenesis, it was reported that Myc-driven tumorigenesis in mice depended heavily on increased protein translation induced by Myc through enhanced ribosome biogenesis; restoring protein translation levels to "normal" through ribosomal protein haploinsufficiency significantly impaired Myc's oncogenic potential (141).

Direct inhibition of ribosome biogenesis has shown promising results for cancer treatment. CX-5461, a small molecule inhibitor of Pol I transcription, is currently in clinical trials for cancer therapy. Its ability to selectively inhibit rRNA transcription has been shown to significantly decrease cancer cell proliferation in vitro and tumor formation in xenograft mouse models (142) through induction of senescence and autophagy. Furthermore, these anti-proliferative effects seem to be limited to cancer cell lines, while proliferation of normal cells remained unaffected. Analysis of p53 status of drug sensitive cancer cells revealed that in general, cancer cell sensitivity to CX-5461 was similar between p53 wild type and mutant contexts, except in hematologic malignancies where cell lines with wild type p53 status showed a higher sensitivity to the drug. In case of Myc-induced B-cell lymphomas in mouse models, CX-5461 induced apoptosis through stabilization of p53. Therefore, CX5461 appears to be a promising new therapeutic strategy for cancer treatment. However, as the anti-proliferative functions of CX-5461 include induction of senescence and senescence has been shown to induce inflammatory response with potential pro-tumorigenic functions (143-145), the long-term effects of CX-5461 in cancer therapy need to be monitored closely to establish its success as a therapeutic agent.

As a further cautionary note in development of drugs targeting ribosome biogenesis for cancer therapy, certain ribosomal components have also been shown to function as haploinsufficient tumor suppressors. In zebrafish, heterozygous deletion of a set of 11 ribosomal

proteins led to development of peripheral nerve sheath tumors, suggesting a tumor suppressive role for these ribosomal components (146). Further analysis of tumor cells derived from these zebrafish revealed that mutations in these ribosomal proteins led to a repression of p53 mRNA translation in a selective manner (147). These results suggest that alterations in ribosome biogenesis and expression of ribosomal components can contribute to tumorigenesis potentially through both global upregulation of protein synthesis to sustain enhanced proliferative rates as well as through a more selective translational output. These results also signify the importance of selecting appropriate targets within the ribosome biogenesis pathway for cancer therapy. Further understanding of the mechanisms by which ribosome biogenesis pathway can contribute to tumorigenesis is necessary for identifying the genetic contexts that would respond the best to therapies targeting this pathway.

The ARF tumor suppressor pathway has a deeply ingrained role in negatively regulating multiple aspects of ribosome biogenesis. The work presented in this thesis sought to identify novel modes of action by which ARF-dependent regulation of ribosome biogenesis could contribute to cellular transformation. Hence, the next few sections will focus on detailing the ARF-mediated regulatory component of ribosome biogenesis pathway.

1.8 ARF Tumor Suppressor and Its Mode of Action

The CDKN2A (INK4A/ARF) locus, located on the p-arm of human chromosome 9, encodes for two tumor suppressor proteins, p16INK4A and p14ARF (p19ARF in mice). The mRNA transcript of each protein has a different first exon—exon 1α for INK4A and exon 1β for ARF—but share exons 2 and 3, with ARF splicing into the exon 2 in an alternate reading frame from INK4A (hence the name ARF) (Figure 1.1). This splicing event leads to generation of

proteins with distinct amino acid sequences despite a similar nucleotide sequence (148,149). The INK4A protein inhibits cell cycle progression in an Rb-dependent manner (42). Passage of cells through the G1 phase is regulated by a hypophosporylated Rb protein, which binds the E2F transcription factor and prevents transcription of the S-phase genes. This inhibition is lifted by phosphorylation of Rb by the CyclinD-CDK4/6 complex. INK4A prevents CDK-mediated phosphorylation of Rb, thereby promoting Rb-dependent inhibition of S-phase entry. P14ARF was originally thought to be a redundant transcript with no translated protein. However, further study of this alternately spliced variant showed that, like INK4A, ARF is capable of inducing cell cycle arrest, the difference being that ARF lifts inhibition over the p53 tumor suppressor instead of Rb (150). ARF is capable of binding to the E3 ubiquitin ligase, MDM2 (HDM2 in humans), and sequester it to the nucleolar compartment, thereby preventing MDM2-dependent ubiquitination and subsequent degradation of p53 protein (120,151-153). The stabilized p53 protein is then capable of functions in inhibiting cell cycle progression (Figure 1.1).

For a long time, ARF's tumor suppressive roles were thought to be limited to activation of p53. If this were the case, loss of ARF would essentially phenocopy loss of p53. However, studies done in *TP53*-/- and *Arf* -/- mouse models show varying results, suggesting ARF to have functions outside of p53 activation. Majority of the *TP53* -/- mice developed lymphomas (71%) with a few sarcomas (154), while the *Arf* -/- mice developed a wide range of tumors including sarcomas (49%), lymphoid tumors (29%), carcinomas (17%) and tumors of the nervous system (11%) (155). Furthermore, *TP53* -/-; *Arf* -/- double knockout mice developed a broad spectrum of tumors, many of which were not observed in the *TP53*-/- or *Arf*-/- mice, and the incidence of multiple primary tumors within an animal were higher in the double knockout mice (156). These results strongly indicate that ARF performs critical functions in more than just the p53 pathway.

Further studies on tumor suppressive functions of ARF revealed that in addition to inducing p53-dependent G1 cell cycle arrest, ARF is also capable of inducing G2 cell cycle arrest in tumor cells deficient of p53 function (157). ARF can inhibit activity of CDC2, the cyclin-dependent kinase involved in entry of cells into mitosis, by suppressing CDC25, the upstream activator of CDC2; ARF can further induce expression of the CDK inhibitor, p21. Further analysis of the G2 check point induced by ARF revealed that ARF induction leads to the activation of CHK2-dependent DNA damage response pathway, which culminates in inhibition of CDC2 and mitotic entry (158). Induction of ARF was shown to lead to stabilization of the histone acetylase TIP60, which in turn activated the ATM kinase, an important mediator of the DNA damage response pathway and its downstream target CHK2. TIP60 is capable of activating ATM in an acetylation-dependent manner (159), and ARF appears to utilize this function of TIP60 to activate the ATM-dependent G2 checkpoint. Particularly in cases of genotoxic stress, both ARF and TIP60 are required for activation of the ATM/CHK2 response and the subsequent G2 cell cycle arrest. Interestingly, ARF has also been reported to activate G1 checkpoint in a p53-independent manner, where ectopic expression of ARF or induction of ARF through genotoxic stress led to an accumulation of Rb protein (160). Mechanistically, it was shown that Rb is targeted for proteasomal degradation upon acetylation of Rb in its C-terminus region by TIP60. Induction of ARF was shown to suppress TIP60-mediated acetylation of Rb, thereby stabilizing Rb protein and inducing a G1 checkpoint. How ARF promotes TIP60-dependent acetylation of ATM while preventing TIP60-dependent acetylation of Rb remains to be tested. Given the ability of ARF to regulate subcellular localization of its target proteins, as was demonstrated in the case of nucleolar sequestration of MDM2, it is possible that while stabilizing TIP60 on one hand, ARF is also capable of regulating its subcellular localization, thereby preventing its access to some proteins

while promoting its activity on others. This kind of multimodal functioning of ARF is evident in the strong inhibitory role it plays in the ribosome biogenesis pathway.

Hyperproliferative signals, as those induced by overexpression of oncogenes, have been shown to induce ARF expression (161-166). Activated ARF then participates in inhibiting proliferation of these oncogene expressing cells through multiple mechanisms, including suppression of the ribosome biogenesis pathway. Several studies have shown that ARF is capable of suppressing rDNA transcription through different mechanisms. Ayrault et.al., have shown that ARF inhibits phosphorylation of the rDNA transcription factor, UBF1, a modification necessary for UBF1's transcriptional functions (167). Alternately, ARF was also shown to bind the nucleolar localization sequence of another rDNA transcription factor, TTF1, and prevent its nucleolar localization, there by suppressing its transcriptional functions (168). P19ARF has been shown to inhibit nucleolar localization of the DEAD box RNA helicase, DDX5, which has critical functions in both rDNA transcription as well as maturation of rRNA precursors (161). ARF is also capable of sequestering and promoting degradation of the nucleolar protein, NPM, which has been shown to have critical functions in rDNA transcription, rRNA maturation and shuttling of pre-ribosomal subunits to cytoplasm (123,169-173).

An additional level of regulation employed by ARF to inhibit oncogenic transformation involves a direct control of oncogenes themselves. ARF has been shown to suppress the transcriptional activity of E2F factors 1-3, potentially by forming a repressive ARF-E2F complex over E2F consensus sequence at promoters of E2F target genes (163). ARF also binds c-Myc and inhibits its transactivation functions in a similar manner (174). Additionally, ARF was reported to sequester cMyc's transcriptional cofactor, DDX5, away from promoters of c-Myc target genes (165); in absence of DDX5, transcriptional activity of Myc was significantly reduced. Additionally,

ARF was also shown to directly bind c-Myc and cause it to relocate from nucleoplasm to nucleolus, thereby suppressing its transcriptional activity at specific target genes (175).

In addition to the critical role ARF plays in regulating synthesis of ribosomes, multiple studies have indicated that ARF is also capable of regulation translation of select mRNAs. Kawagishi et.al., found that induction of p19ARF led to a decreased vascular endothelial growth factor A (VEGFA) protein levels. Further analysis of this inhibition revealed that overexpression of p19ARF did not alter the overall VEGFA mRNA levels, but significantly decreased the association of VEGFA mRNA with actively translating polyribosomes (176). P19ARF was further shown to regulate translation of DHX33 and Drosha mRNAs in a similar manner (177,178). It was previously reported that induction of p14ARF led to a decrease in the initiation factor eIF2, which is necessary for binding of the initiator tRNA to the 40s ribosomal subunit (179). While this could potentially be the mechanism employed by ARF to suppress mRNA translation, how ARF achieves selectivity in this process remains to be tested.

To further expand the list of cellular processes regulated by ARF, study of eye development in *Arf* null mice revealed ARF to have anti-angiogenic functions (180). Regression of the hyaloid artery is a critical part of normal ocular development and *Arf* null mice showed abnormal vasculature in the retrolental tissue due to failed regression of the hyaloid artery. Further analysis of this developmental process revealed that ARF induces the regression of ocular vasculature through its regulation of the PDGFRβ signaling (181). ARF suppresses expression of PDFGRβ in both transcriptional and protein stability manner, thereby preventing abnormal retention of vascular structures in the vitreous body (182,183). Mechanistically speaking, regression of the hyaloid vasculature has been known to occur through apoptotic cell death of endothelial cells and pericytes (184,185). While ARF expression hasn't been linked directly with cell death during

development of the eye, studies done in different cancer cell lines revealed that ARF could induce apoptosis through multiple mechanisms.

The p32 protein, which has been shown to have critical functions in maintaining mitochondrial structure and functions (186), was also demonstrated to bind ARF in its C-terminal region and promote ARF's translocation to mitochondria (187). This mitochondrial ARF was able to induce reduction of mitochondrial membrane potential and release of cytochrome C in a p53-dependent manner. Furthermore, cancer-associated mutations found in the p32-binding region of ARF have been shown to impede binding of ARF with p32, and prevent mitochondrial localization and pro-apoptotic functions of ARF. In addition to the p53-dependent induction of apoptosis, ARF has also been reported to induce apoptosis in a p53-independent manner through inhibition of anti-apoptotic factors, Mcl-1 and Bcl-xL, and activation of the pro-apoptotic factor, Bak (188-190). As an additional mechanism, ARF overexpression has also been shown to induce expression of the pro-apoptotic factor, PUMA, both at the mRNA and protein levels (191). The ability of ARF to induce apoptosis was significantly attenuated upon loss of PUMA expression, suggesting that ARF functions through PUMA to induce apoptosis. On the contrary, loss of the cell cycle regulator, p21, led to a strong enhancement of ARF-mediated cell death. Treatment of ARF overexpressing cells with a pan-caspase inhibitor, z-VADfmk, completely attenuated ARF-dependent DNA fragmentation/cell death. However, z-VAD treatment of p21 deficient cells overexpressing ARF only managed to decrease the DNA-fragmentation levels down to those observed in ARF overexpressing cells instead of a complete inhibition of DNA fragmentation. These results suggest that in a p21-deficient background, ARF is capable of inducing DNA fragmentation/cell death in a caspase-independent manner. Once such caspase-independent cell death mechanism is autophagy, where cells metabolize cellular components such as mitochondria through lysosomes

to generate the macromolecules necessary for cell survival, as well as for clearance of damaged organelles in conditions of nutrient depravation (192).

The Exon 2 region of ARF has been implicated in induction of autophagy, with an internal methionine start site in Exon 1β of ARF coding for a small ARF (smARF) variant which exclusively localizes to mitochondria and is capable of inducing mitophagy (autophagy of mitochondria) (193). Furthermore, tumor-associated mutations in Exon 2 of CDKN2A locus that affect ARF, but not INK4A, were shown to inhibit ARF's ability to induce autophagy, suggesting a potential tumor suppressive role for ARF-induced autophagy. ARF mitochondrial localization was further shown to require its association with the heat-shock protein, HSP70 (194), and ARF induces autophagy by inhibition Bcl-xL mediated suppression of autophagy-inducting complex, Beclin-1/Vps34 (195).

From its ability to induce cell cycle arrest to promote cell death, ARF expression can function in anti-proliferative manner (Figure 1.2). Mobilization of ARF in response to oncogenic stimuli and/or compromised nucleolar functions makes its anti-proliferative functions particularly important for preventing aberrant cell proliferation and survival. Hence, identifying mechanisms regulating ARF's expression can give us a deep insight into pathways regulating normal and aberrant cell proliferation.

1.9 Regulation of ARF Expression

While the far-reaching anti-proliferative effects of ARF are extremely desirable in preventing aberrant cell proliferation, overstimulation of ARF under normal conditions can be functionally counterproductive both at the cellular and organismal levels. For this reason, the p14ARF promoter is silenced through methylation under normal cellular conditions. Robertson et.al., found that the p14ARF mRNA expression significantly increased upon treatment of cells

with the methylation inhibitor, 5-aza-29-deoxycytidine (196). Using a reporter construct, they were further able to identify regulatory elements present within the ARF promoter region and found that p53 overexpression repressed ARF's promoter activity. Given ARF's ability to activate p53, this repression was indicative of a negative feedback loop that functions in regulating ARF expression. Bracken et.al., later showed that the Polycomb Repressive Complex 2 (PRC2) is critical for suppression of the CDKN2A locus in response to overexpression of the oncogene Bim1. Furthermore, overexpression of p16INK4A and p14ARF in senescent cells is dependent on down-regulation of the EZH2 component of the PCR2 complex. It was further reported that the histone dacytelase (HDAC) and the polycomb repressive complex 1 (PRC1), were both capable of suppressing the p14ARF locus (197,198). Further study of the p53-dependent repression of the ARF promoter showed that p53 was capable of binding and recruiting both HDAC and polycomb group proteins to the p14ARF locus to suppress ARF expression (199). Additionally, certain oncogenes, such as E2F3, have also been shown to suppress ARF transcription to regulate both normal and abnormal cell proliferation (200).

In addition to transcriptional regulation, ARF has also been shown to be regulated through ubiquitination-mediated protein degradation. Kuo et.al., reported that ARF protein undergoes proteasomal degradation by means of N-terminal polyubiquitination, which is dependent on the ubiquitin activating E1 enzyme (201). Furthermore, overexpression of NPM was reported to stabilize ARF protein. As a means of identifying the E3 ligase responsible for ubiquitination of ARF, Chen et.al., isolated NPM-protein complexes and through mass spectroscopic analysis, identified a protein, ULF (ubiquitin ligase for ARF), which had a potential ubiquitin ligase domain (202). RNAi mediated knockdown of ULF was shown to significantly stabilize ARF protein, and ULF-mediated ubiquitination of ARF was antagonized by both Myc and NPM. These results

confirmed a role for ULF as NPM-regulated E3 ligase with ability to target ARF for proteasomal degradation. The ability of Myc to stabilize ARF once again supports ARF's role in surveillance of oncogenic signals.

As expression of oncogenes such as E2F and Myc is necessary for normal cell proliferation, ARF induction requires a means to distinguish expression of oncogenes under normal cellular conditions versus conditions of cellular transformation. In case of Myc, it was shown that low level of Myc expression (as can occur during normal cell proliferation) has no effect on ARF protein levels. However, high levels of Myc expression leads to stabilization of ARF protein through suppression of ULF-mediated ARF ubiquitination (166). In the case of E2F, a non-canonical E2F responsive element (EREA) was reported in the promoter region of p14ARF. This EREA element was further shown to be transcriptionally active in presence of aberrant E2F expression (as achieved through E2F overexpression or inhibition of pRb) but not under normal cell proliferative conditions (203). It was further reported that this EREA had a significantly weaker binding affinity to E2F when compared to canonical E2F site, potentially requiring a greater level of E2F expression to initiate transcription from the EREA site. Thus, the Myc-ULF-ARF protein stability axis and the E2F-EREA-ARF transcriptional regulation provide important links in understanding the mechanisms utilized by cells to govern normal cellular functions while suppressing aberrant cell proliferation.

Given the extent of inhibitory functions ARF plays in cell proliferation, suppressing expression of ARF under conditions requiring rapid cell proliferation (as required during embryogenesis) is extremely critical. As such, transcription factors with critical functions during embryonic development have been shown to function in repression of ARF. One such factor is TBX2, a member of the T-box family of transcription factors, which has been shown to function

in development of multiple organs, including mammary glands, kidneys, heart and lungs (204,205). As such, potentially as a means to suppress unwanted growth-inhibitory signals during development, TBX2 has been reported to strongly inhibit expression of ARF, INK4A as well as INK4B in a transcription-dependent manner (206,207). Furthermore, ectopic expression of TBX2 is capable of negating oncogene-dependent activation of ARF, stressing a potent inhibitory role for TBX2 in ARF expression, a role which can potentially be exploited for enhancing aberrant cell proliferation.

In addition to suppressive mechanisms preventing ARF from functioning in a growth-suppressive manner during development, mechanisms that positively regulate ARF expression during development have also been discovered. As mentioned in the previous section, expression of ARF is necessary for proper eye development during embryogenesis. The TGFβ signaling has been implicated in inducing expression of ARF to promote regression of the hyaloid vasculature in developing eye (208). TGFβ signaling leads to remodeling of the ARF promoter region by Smad2/3 complex and a subsequent enhancement of Pol II-driven transcription of ARF (209). Tight regulation of ARF expression (both positive and negative), therefore, has significant developmental roles in embryogenesis as well as supervisory roles in preventing abnormal cell functions in developed organisms. Accordingly, genetic alterations that impinge on these regulatory mechanisms can have deleterious effects on cellular and organismal functions, as proven in the case of tumorigenesis.

1.10 Functional Relevance of ARF In Cancer

Deletion of the CDKN2A locus has been reported to be a common occurrence in multiple cancers (Figure 1.3). In addition to genomic deletions, methylation-driven silencing of the locus

has also been reported as a potential mechanism promoting tumorigenesis (197,210-220). For a long time, alterations in the CDKN2A locus and their contribution to tumorigenesis were predominantly ascribed to loss of p16INK4A functions. However, studies are now being conducted to determine how alterations in CDKN2A locus can contribute to p14ARF functions. A germline deletion in exon 1beta of CDKN2A gene, specifically effecting p14ARF but not INK4A, was reported in a family, predisposing its members to melanoma and neural stem tumors (221). Analysis of p16INK4A and p14ARF methylation status in 100 breast, 95 colon and 27 bladder cancers revealed that compared to matched normal tissue, tumor samples showed hypermethylation of both INK4A and ARF genes in all three cancer types (219). Interestingly, hypermethylation of ARF occurred at higher rates in all three cancers compared to hypermethylation of INK4A, although aberrant methylation in both genes correlated with a significantly poorer outcome in all three cancer types. In case of colorectal cancers, p14ARF gene silencing in absence of alterations in p16 expression was shown to be strongly associated with tumor tissue as opposed to normal tissue (211,214,222). In laryngeal squamous cell carcinoma, patients with high ARF mRNA expression were shown to have better survival outcome than those with low ARF expression (223). Expression pattern of INK4A and ARF in melanomas showed a strong reduction of both ARF and INK4A mRNA levels in melanoma samples compared to normal tissue, with an inverse correlation between INK4A/ARF mRNA expression and melanoma thickness (224). Analysis of melanoma cell lines revealed an acetylation-dependent silencing of ARF and a methylation-dependent silencing of INK4A locus. Furthermore, overexpression of either gene alone was able to significantly suppress melanoma cell proliferation, migration and invasion, while co-expression of both genes led to a more profound suppression of malignant properties of cells, suggesting an important biological role for both of these tumor suppressors in

melanoma. ARF status in laryngeal squamous cell carcinoma revealed that patients with late stage tumors showed low ARF expression and had significantly decreased overall survival when compared to patients with high ARF expression (223).

In addition to correlational studies, functional studies of p14ARF are highlighting significant biological implications for ARF loss in promoting tumor progression. Functional contribution of P14ARF in glioblastomas, one of the tumor types with a high incidence of CDKN2A deletions, revealed that ARF suppresses angiogenesis in human glioma cell lines by upregulating tissue inhibitor of metalloproteinase-3 (TIMP3) (225). Functional relevance for ARF in hepatocellular carcinoma (HCC) came from the observations that high levels of ubiquitin-specific protease 7 (USP7) expression strongly correlate with tumor size, microvascular invasion and tumor differentiation (226). Furthermore, HCC patients with high USP7 expression also showed poor overall survival rates and a higher incidence of tumor recurrence. Functional studies in HCC cell lines revealed that USP7 promoted cancer cell proliferation through degradation of p14ARF (227). ARF is also reported to function in suppressing growth of chemo-resistant non-small cell lung cancers by inducing apoptosis through mitochondrial dysfunction (228).

The extensive tumor suppressive roles performed by ARF make it a potential target for cancer therapy. In fact, Saadatmandi et.al., tested this hypothesis by expressing replication deficient adenoviral vectors encoding the exon 1beta of ARF in multiple cancer cell lines with varying p53 and ARF status (229). Their results were promising in that exon 1beta expression was able to suppress proliferation of cancer cells with both wild type and mutant p53 status. One caveat for perusing this avenue for cancer therapy is that a few cases of cancers have been reported where aberrant elevation in ARF expression appears to promote tumorigenesis rather than suppress it. In a recent study of 1080 patients with invasive ductal carcinoma of breast, it was reported that

patients with tumors containing elevated ARF expression had a greater than three-fold increased risk of recurrence when compared to patients with ARF-negative tumors (230). The functional contribution of elevated ARF expression toward breast tumorigenesis is yet to be determined. However, in pancreatic cancer, ARF expression was shown to stabilize the transcription factor, SLUG, and promoted epithelial-mesenchymal transition (EMT) by repressing E-cadherine and increasing matrix metalloproteinase 7 (231,232). Expression levels of SLUG, MMP7 and ARF were further shown to be positively correlated in human prostate cancer samples. These results indicate that complex mechanisms govern ARF's cellular functions, which can act in tumor suppressive or oncogenic manner based on the genetic context of cells (Figure 1.2). Further delineating the ARF's functional pathway can give us a better insight into mechanisms by which ARF regulates normal and aberrant cell behavior and the cellular context that allows for utilization of ARF regulatory network in targeted cancer therapy.

For the purpose of this thesis, I am going to focus the rest of this chapter on the ribonuclease Drosha, a novel target of ARF reported a few years ago. Kuchenreuther and Weber reported that the ARF tumor suppressor was able to inversely regulate Drosha mRNA translation in mouse cells (178), where loss of ARF increased Drosha protein levels through an enhancement of Drosha mRNA translation. ShRNA mediated knockdown of Drosha in an *Arf -/-* context was further shown to impair ribosome biogenesis as well as Ras-mediated transformation of the *Arf -/-* cells. However, how elevated Drosha levels in *Arf* deficient cells contribute to rRNA processing and the role this rRNA component of Drosha's functions plays in cell proliferation and transformation remains to be tested. As the work presented in chapters 2 and 3 of this thesis help to delineate the mechanism by which ARF regulates Drosha's functions, the next few sections will provide information on Drosha and what is known of its cellular functions.

1.11 Multifaceted Functions Of Drosha Ribonuclease

Drosha is an RNAse III endonuclease capable of cutting double-stranded RNAs. Human Drosha is a 1,374 amino acid protein, encoded by the *RNASEN* gene located on the p-arm of chromosome 5. Drosha contains multiple domains capable of protein-protein and protein-nucleic acid interaction, double stranded RNA (dsRNA) cleavage and dsRNA binding functions (Figure 1.4). Isolation of Drosha-containing protein complexes revealed that Drosha precipitated in two distinct complexes containing different groups of proteins: the higher molecular weight Drosha complex contained proteins belonging to the DEAD-box RNA helicase family (DDX17/P72), Ewings sarcoma family of proteins (EWS), and heterogenous nuclear ribonucleo proteins (hnRNPM4), while the lower molecular weight Drosha complex contained only Drosha and the DiGeorge Syndrome Critical Region 8 (DGCR8) proteins (233). It was further shown that the Drosh-DGCR8 complex had critical microRNA (miRNA) processing functions (233-235). miRNAs are small non-coding RNAs which function in post-transcriptional gene silencing. MiRNA biogenesis involves transcription of primary miRNA transcript by RNA polymerase II (Pol II), which tend to fold into stem-loop structures, which are recognized and cleaved by Drosha-DGCR8 complex to generate a pre-miRNA transcript in the nucleus. The pre-miRNA is then transported out to the cytoplasm where the cytoplasmic RNase, Dicer, further processes it to generate the mature miRNA. The mature miRNA is then assembled into the RNA-mediated silencing complex (RISC), which is capable of inhibiting translation of target mRNAs, thereby participating in gene silencing (236).

Further analysis of Drosha's cellular functions identified Drosha's ability to cleave mRNAs, the DGCR8 mRNA being the first identified target of Drosha's activity (237-239). Newer reports

have further confirmed that Drosha is capable of cleaving and destabilizing select mRNA targets containing stem-loop structures similar to those seen in the pri-miRNA transcripts (240-243). Interestingly, a recent discovery has attributed a novel RNA-cleavage independent function to Drosha (244). It was shown that Drosha was capable of binding polymerase II through its N-terminal proline-rich and arginine/serine-rich domains. Through this interaction with polymerase II, Drosha was able to regulate transcription of select mRNAs. Drosha has further been shown to participate in the processing of ribosomal RNA (rRNA) (Figure 1.5). In fact, Drosha was originally discovered to be the human RNase III with important functions in rRNA processing, where antisense inhibition of Drosha led to an accumulation of rRNA precursors (245). However, Drosha's role in ribosome biogenesis is amongst the least studied aspects of Drosha's cellular functions.

Drosha's mode of action in RNA processing has been extensively studied with miRNA substrates. It was found that in the case of miRNA processing, Drosha required the presence of its binding partner, DGCR8, to achieve substrate specificity (233). Therefore, the combination of Drosha and DGCR8 is considered to be the minimal Microprocessor complex necessary for miRNA cleavage, with Drosha functioning as the catalytic component of the Microprocessor and DGCR8 functioning in substrate recognition. However, recent studies found that DGCR8 on its own had poor substrate specificity (246), suggesting that the combination of Drosha and DGCR8 is necessary for achieving successful target recognition. Recent reports identified the amino acid residues 390-1365 of human Drosha to be critical for it miRNA processing functions (247). Furthermore, Drosha requires interaction with DGCR8 to achieve structural stability (237), and the microprocessor complex is composed of one Drosha and two DGCR8 molecules (247). In addition to miRNA processing, DGCR8 has also been suggested to function in Drosha-mediated

mRNA destabilization as well as Drosha-dependent transcription (244,248). The core complex of Drosha and DGCR8 has been shown to be sufficient to process miRNAs. However, whether this minimal complex is sufficient to process other RNA species regulated by Drosha is not known. While Gregory et.al., showed that DGCR8 containing Drosha complex had a greater efficiency in processing miRNAs, the functional relevance of the larger Drosna-protein complex (containing the EWS, DDX17 and hnRNPM4) is largely unknown. Members of the DEAD-Box RNA helicase family, (DDX17 and DDX5 specifically), have been shown to associate with the Drosha-DGCR8 microprocessor complex to modulate its miRNA processing functions (249,250). However, it is not known if this modulatory effect is limited to processing of the miRNA species, or also applies to Drosha's RNA processing functions in general. Also, whether Drosha is capable of forming functional complexes in absence of DGCR8 remains to be tested, particularly in context of non-miRNA targets of Drosha.

1.12 Regulation of Drosha Expression And Functions

Drosha is an ubiquitously expressed protein with a cell cycle dependent nucleolar localization (245), although the mechanisms regulating this preferential subcellular localization have not yet been discovered. Both c-MYC and E2F1 oncogenes are capable of upregulating Drosha expression in a transcription-dependent manner (251,252). E2F1 has been shown to induce Drosha expression in response to cisplatin treatment of cancer cells and a subsequent increase in expression of the miRNA-630. Interestingly, decreased expression of miR-630 has been shown to inhibit cell proliferation and increase chemosensitivity to cisplatin treatment (253). Therefore, E2F1-mediated upregulation of Drosha could be a potential mechanism employed by E2F1 to prevent cisplatin-induced cell death, although further enquiry is necessary to test this hypothesis.

In addition to the transcriptional regulation of Drosha by Myc and E2F1, the mTOR pathway—which plays a critical role in integrating environmental cues and cell growth, proliferation and survival—has been shown to negatively regulate Drosha protein expression in mouse cell lines (254). Specifically, activation of mTOR pathway led to decreased Drosha protein levels. Further analysis of this reduction in Drosha protein levels showed that Drosha underwent proteasome-mediated degradation upon mTOR activation, and this degradation was caused by MDM2-dependent ubiquitination of Drosha. Furthermore, it was shown that Drosha levels are regulated by nutrient and amino acid availability; glucose or amino acid depravation leads to an increase in Drosha levels, and this increase appears to function in suppressing starvation-based cell death in a miRNA-dependent manner. In addition to regulation of Drosha mRNA and protein levels, several tumor suppressors and oncogenes, such as p53, BRCA1 and estrogen receptor β (ERβ) have been shown to associate with the Microprocessor to modulate its miRNA processing activity (250,255-257). Unfortunately, none of the above mentioned studies have managed to identify the consequences of Drosha upregulation or functional modulation in non-miRNA pathways regulated by Drosha. Particularly, because many of the proteins involved in regulation of Drosha's expression or function have also been implicated in regulation of ribosome biogenesis, regulation of Drosha by these proteins can have functional contribution towards more than just the miRNA pathway. Therefore, unidimensional focus on the miRNA pathway to assess the consequences of altered Drosha expression or function limits our understanding of how changes in Drosha expression and function can impact cellular properties.

1.13 Alterations in Drosha Expression And Cancer

Both genomic amplification (Figure 1.6), which frequently corresponds with a concurrent increase in Drosha mRNA expression, and post-transcriptional amplification of Drosha expression have been reported in multiple cancers. Sugito et.al., found that Drosha expression was enhanced in a fraction of esophageal cancers, with cancer cell survival dependent on the enhanced Drosha expression (258). Drosha expression has also been reported to be elevated in epithelial skin (259), ovarian (260), lung (261,262), breast (263), cervical (264,265), colorectal (266) and bladder (267,268) cancers. Drosha expression in many of these cancers has been shown to have prognostic value and a strong correlation with disease progression and overall survival (261,262,266). Surprisingly, an equal number of cases, where loss of Drosha expression aids tumorigenesis, have also been reported. Neuroblastoma, renal cell carcinoma, endometrial cancer, nasopharyngeal cancer, and cutaneous melanoma have all been reported to have diminished Drosha expression (269-273). Interestingly, some cases of ovarian and breast cancers were also reported to have decreased Drosha expression as a basis for tumor progression (274,275). This ability of Drosha to act in both tumor suppressive and oncogenic manner suggests that it has a complex mode of action in regulating cell proliferation. How alterations in Drosha expression contribute toward tumorigenesis, however, is not clearly known. The predominant hypothesis is that alterations in Drosha expression alter cellular miRNA profiles, which can act in an oncogenic or tumor suppressive manner. Extensive research has therefore been focused on analyzing miRNA alterations upon Drosha loss and determining their contribution toward tumorigenesis. Unfortunately, how alterations in Drosha impact the non-miRNA pathways in which Drosha functions and how they contribute to tumorigenesis have been ignored to a large extent. Given that Drosha regulates multiple RNA species within the cell, determining whether alterations in Drosha

expression can selectively impact specific pathways or whether they globally alter all of the pathways in which Drosha functions can give us valuable insight into how variations in Drosha expression can have differential influence on cellular properties. As an example, both enhancement of ribosome biogenesis and a global decrease in mature miRNAs have been known to be characteristics of cancer cells (132,276). Given Drosha's role in both of these pathways, a gain of Drosha's function in the ribosome biogenesis pathway can lead to an increase in ribosome biogenesis while a loss of Drosha's function in the miRNA pathway can lead to a loss of mature miRNAs, thereby contributing to tumorigenesis in a dual fashion. Whether Drosha employs such a mechanism in aiding tumorigenesis through different modes of action remains to be tested. Identifying mechanisms regulating Drosha expression and functions, particularly those that have the ability to exert control over a specific arm of Drosha's functions can give us a better understanding of cellular contexts that determine Drosha's mode of action.

1.14 Concluding Remarks and Significance of Dissertation Work

A concept that is recurrent through the study of cancer biology is that the genetic context that causes alterations in any given signaling pathway has a huge impact on how those alterations act—tumor suppressive versus tumor promoting. The literature review presented throughout this chapter further stressed this concept, with ribosome biogenesis, ARF and Drosha all functioning in tumor suppressive or oncogenic manner depending on the genetic context of cells. Therefore, understanding as much as we can about mechanisms regulating normal cell behavior and how aberrations in those mechanisms alter cell behavior can give us a better grasp on therapeutic approaches for diverse genetic backgrounds presented by cancer cells.

The purpose of this dissertation is to delineate a pathway that functionally links ARF, ribosome biogenesis, and Drosha, in the hopes of identifying regulatory nodes that can be exploited for cancer therapy. Given that Drosha has been reported to function in rRNA processing and ARF has the ability to regulate nucleolar localization of multiple proteins involved in ribosome biogenesis, I hypothesized that ARF could regulate Drosha's nucleolar functions by preventing its localization to the nucleolus. Chapter 2 focuses on delineating ARF's role in nucleolar functions and its regulation of Drosha expression as pertaining to nucleolar compartment. As Drosha amplification is a common event in cancer cells, we further analyzed how Drosha overexpression could contribute to cellular transformation, paying particular attention to alterations in the nucleolar compartment upon Drosha overexpression. Chapter 3 attempts to identify Drosha's functional relevance in the nucleolar compartment, with further mechanistic revelations on ARF-Drosha axis. Chapter 4 summarizes the data and provides avenues for furthering our understanding of this ARF-Drosha-ribosome biogenesis pathway and its potential use in targeted cancer therapy.

1.15 FIGURES

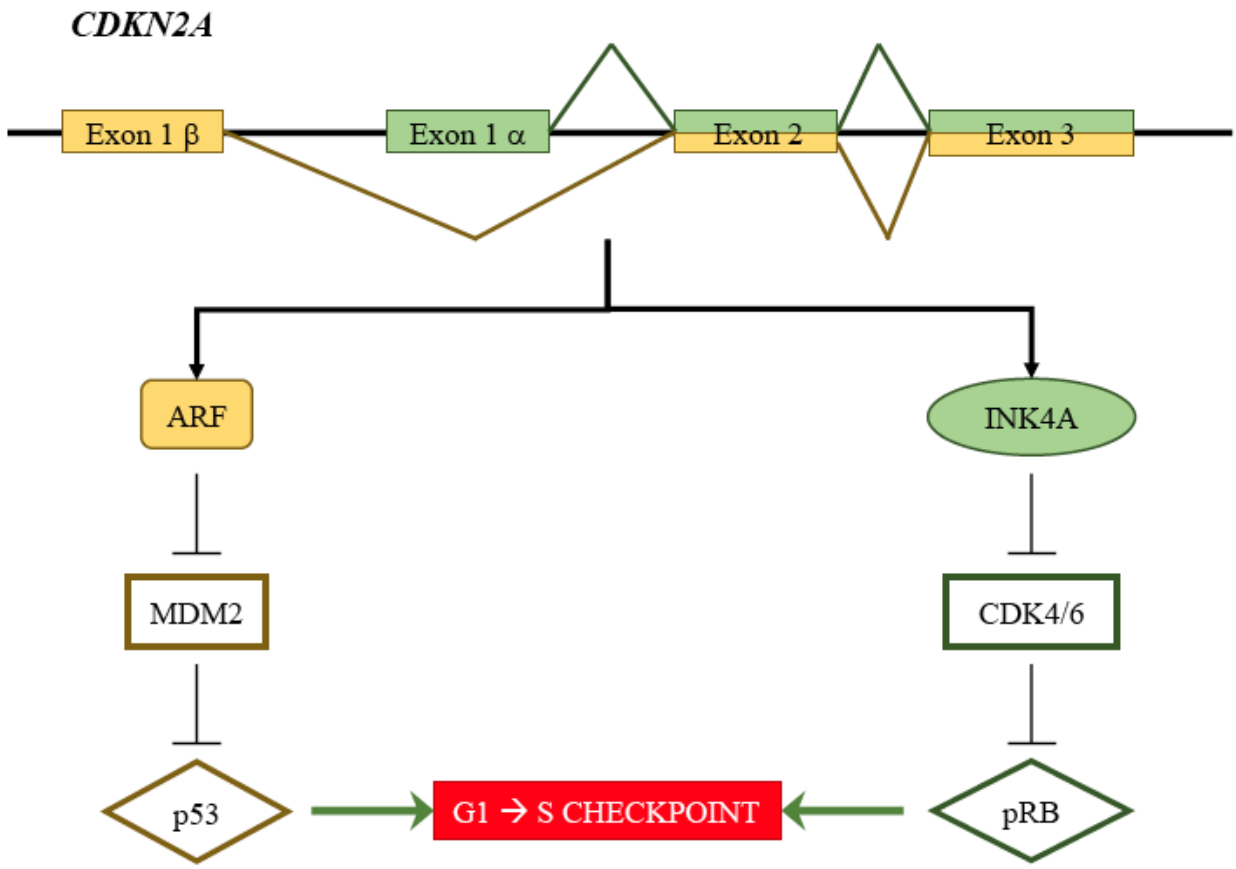


Figure 1.1 – CDKN2A locus and gene products. The CDKN2A locus is depicted as the horizontal bold line, with exons encoding ARF (yellow) and INK4A (green) depicted as rectangular boxes. the exon 1β of ARF splices into exon 2 in an alternate reading frame from INK4A exon 2, thereby generating a protein with little homology to INK4A. The ARF and INK4A proteins suppresses transition of cells from G1 to S phase through activation of p53 and Rb pathways as depicted.

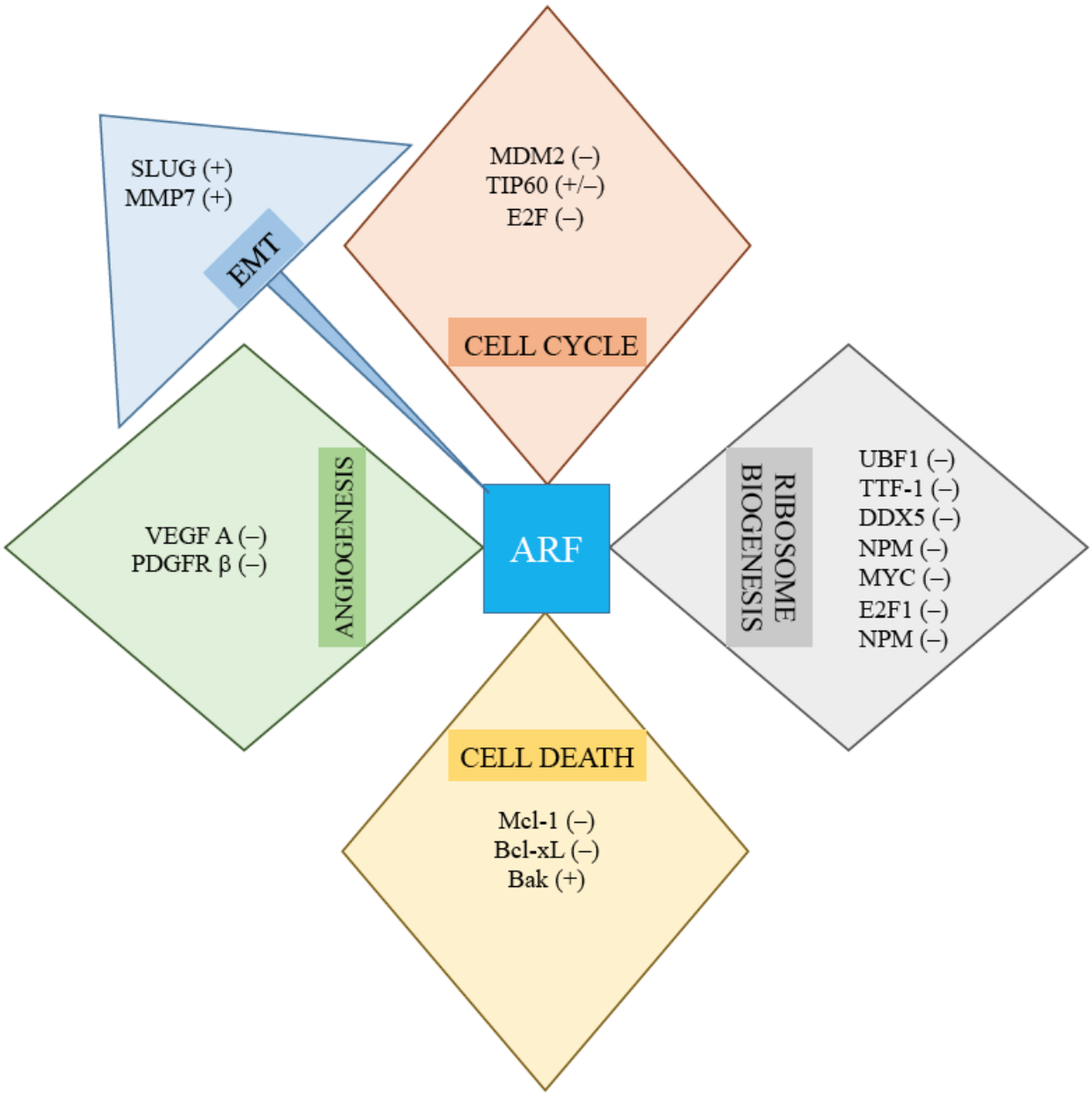
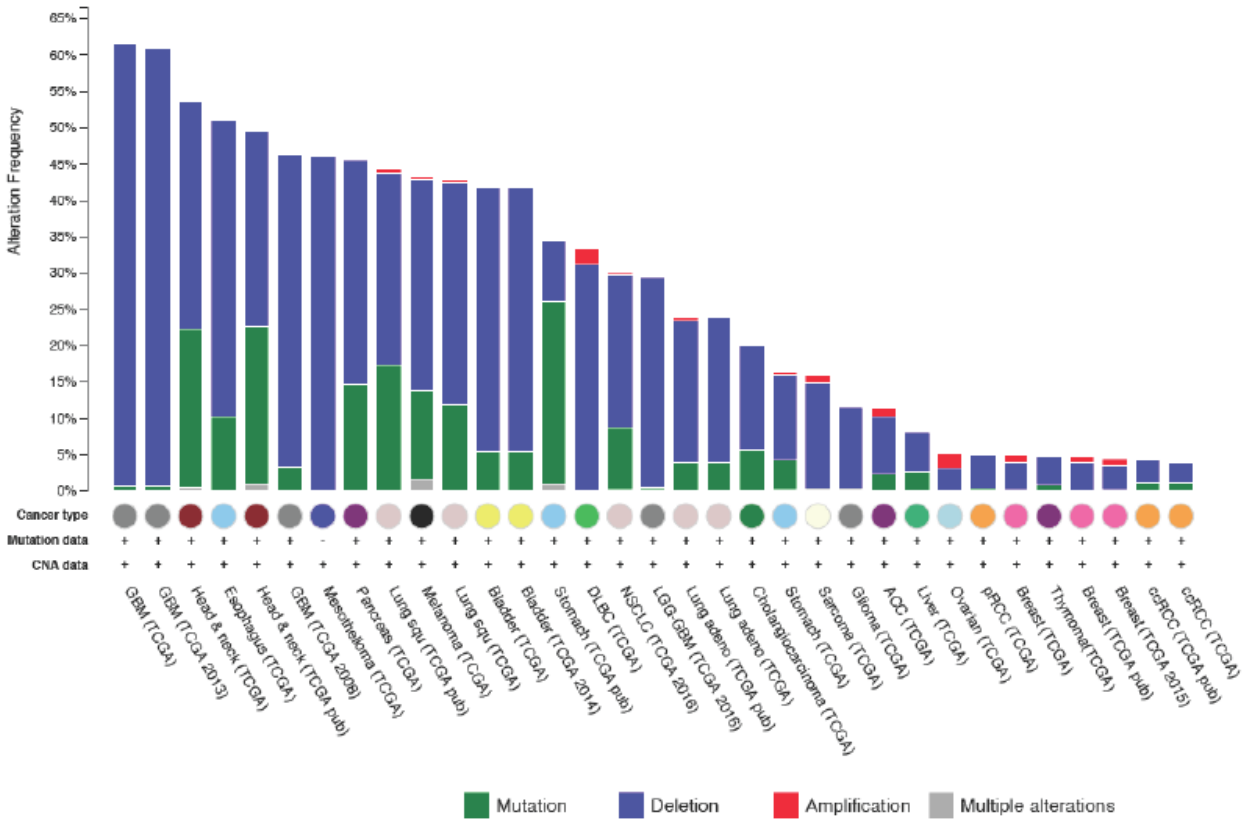

SLUG (+)
MMP7 (+)
EMT
MDM2 (–)
TIP60 (+/–)
E2F (–)
CELL CYCLE
VEGF A (–)
PDGFR β (–)
ANGIOGENESIS
ARF
RIBOSOME BIOGENESIS
UBF1 (–)
TTF-1 (–)
DDX5 (–)
NPM (–)
MYC (–)
E2F1 (–)
NPM (–)
CELL DEATH
Mcl-1 (–)
Bcl-xL (–)
Bak (+)

Figure 1.2 – Tumor suppressive and oncogenic functions of ARF. ARF suppresses tumorigenesis through its negative regulation of cell cycle, ribosome biogenesis, and angiogenesis as well as through positive regulation of the apoptotic pathway. ARF also acts in oncogenic manner through positive regulation of EMT-inducing factors. The protein targets of ARF in each regulatory network are indicated with positive versus negative regulation indicated as (+) or (–). ARF-dependent regulation includes mechanisms of transcriptional regulation, protein stability and subcellular localization.



<u>Figure 1.3</u> – Genomic alterations in CDKN2A locus in different cancer. Analysis of genomic alterations at the CDKN2A locus in different cancer types, generated by the TCGA research network (www.cbioportal.org). The CDKN2A locus is predominantly deleted in multiple cancers, with glioblastomas showing a loss of this locus in approximately 60% of cases.

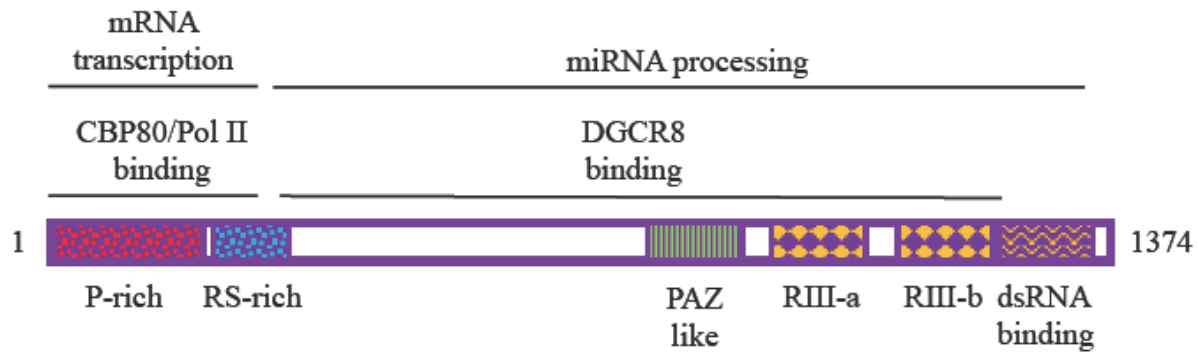


Figure 1.4 – Domain composition of Drosha. Drosha has been reported to function in biogenesis of multiple RNA species within the cells through protein-protein and protein-nucleic acid interactions. The domains necessary for mRNA transcription and miRNA processing are depicted along with the DGCR8-binding region. The P-rich (Proline-rich) and RS-rich (Arginine/Serine rich) regions are important for Drosha's ability to bind CBP80 and RNA Polymerase II. The RIII-a and RIII-b are the RNase III domains and the dsRNA binding stands for double stranded RNA binding region.

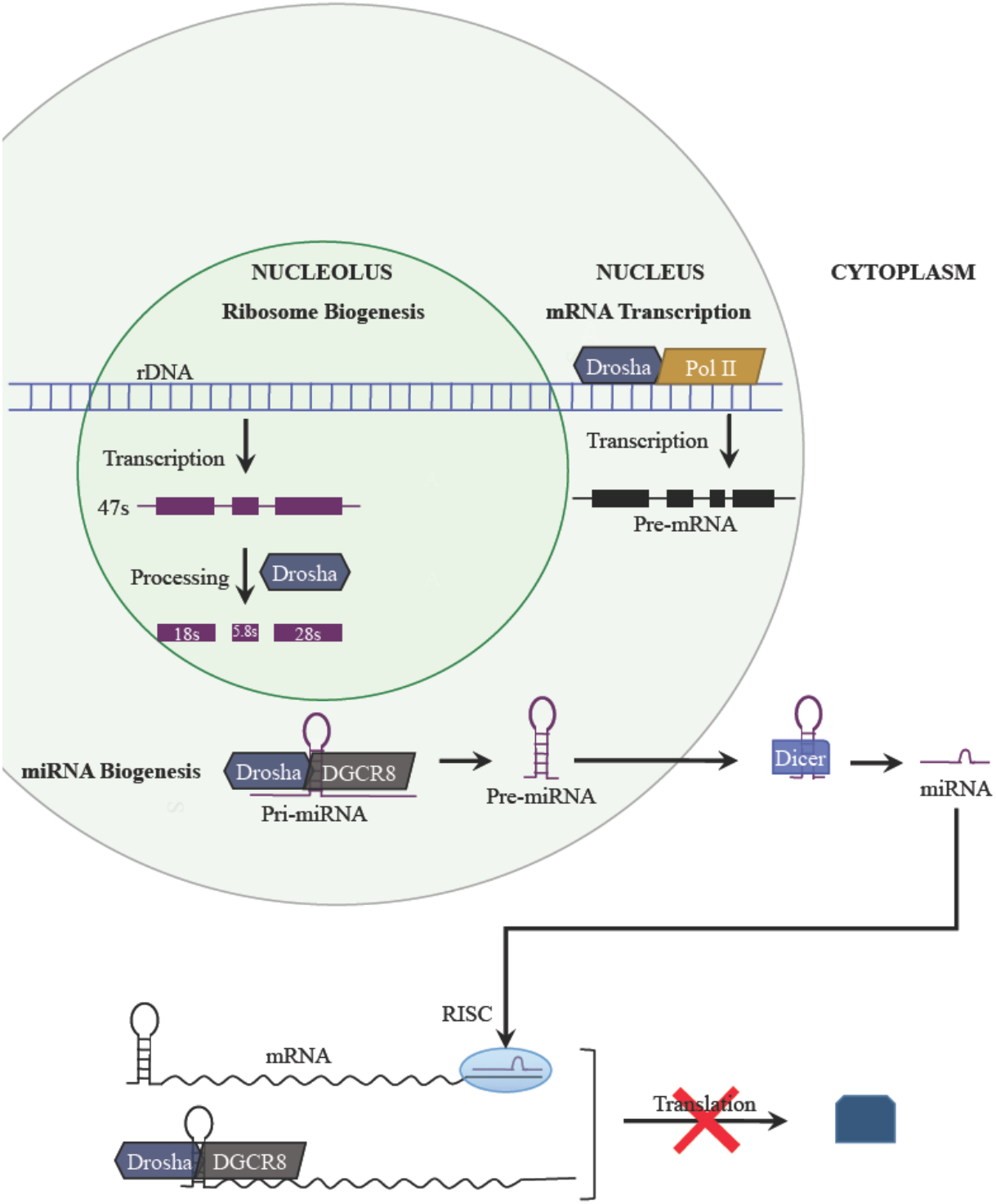

NUCLEOLUS
Ribosome Biogenesis
NUCLEUS
mRNA Transcription
CYTOPLASM
rDNA
Drosha
Pol II
Transcription
47s
Pre-mRNA
Processing
Drosha
18s
5.8s
28s
miRNA Biogenesis
Drosha
DGCR8
Pri-miRNA
Pre-miRNA
Dicer
miRNA
RISC
mRNA
Translation
Drosha
DGCR8

Figure 1.5 – Summary of Drosha's cellular functions. Drosha functions in biogenesis of multiple RNA species within the cells, including rRNA, miRNA and mRNA. While Drosha participates in the rRNA and miRNA biogenesis pathways as a ribonuclease, it was shown to regulate select group of mRNAs in a transcriptional manner, through its ability to bind RNA Polymerase II. Drosha's RNA cleavage functions were discovered to be dispensable for mRNA transcription. Drosha-DGCR8 complex also regulates gene expression through direct cleavage of select group of mRNAs.

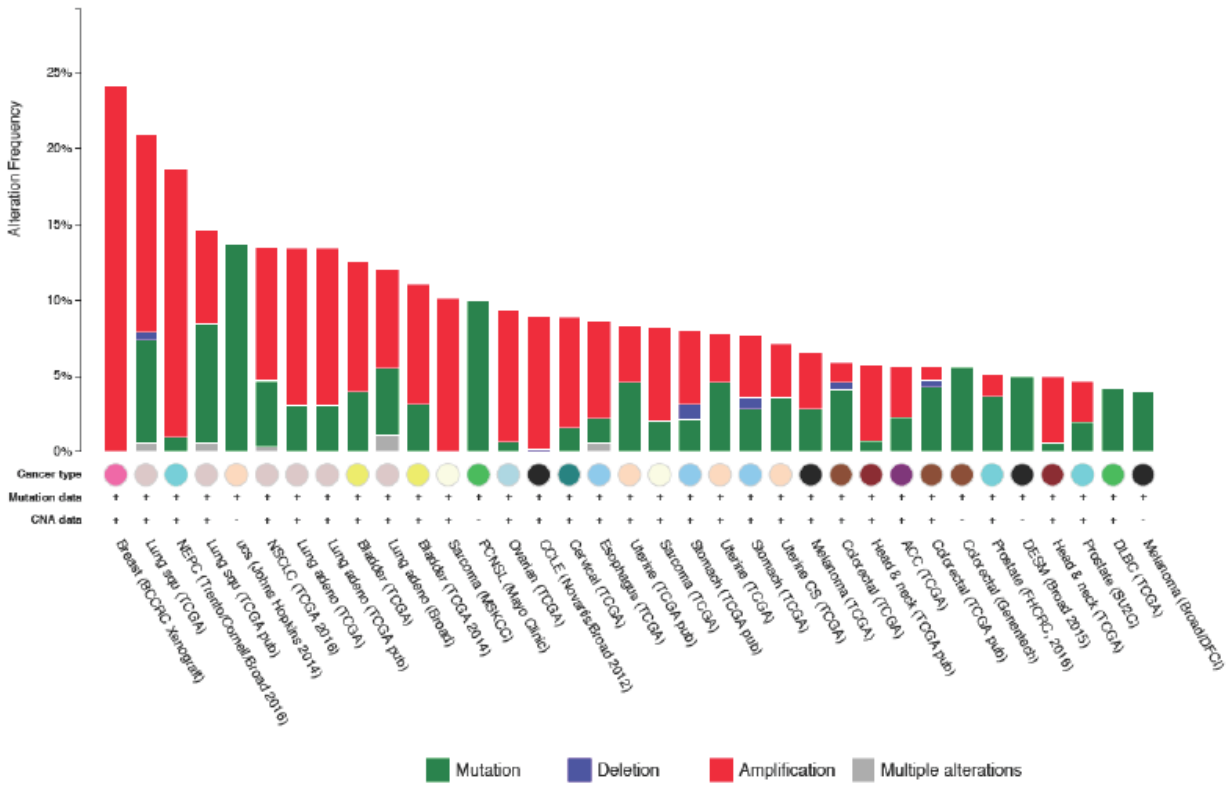


Figure 1.6 – Genomic alterations of *RNASEN* locus in cancer. Alterations in the *RNASEN* genomic locus, encoding the human Drosha protein, were analyzed in multiple cancers using the TCGA research network (www.cbioportal.org). Genomic amplification of Drosha is observed in multiple cancers.

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CHAPTER 2:

THE ARF TUMOR SUPPRESSOR REGULATES RIBOSOME BIOGENESIS THROUGH INHIBITION OF DROSHA EXPRESSION AND NUCLEOLAR LOCALIZATION

2.1 ABSTRACT

ARF is a tumor suppressor protein encoded by the *CDKN2A* locus and is induced by numerous oncogenic stimuli. Upon its induction, ARF inhibits cell proliferation through p53-dependent cell cycle arrest or apoptosis. Additionally, ARF prevents cell proliferation and growth via p53-independent mechanisms. These p53-independent properties of ARF have been the intense focus of recent studies, identifying numerous steps in ribosome production that are antagonized by ARF, including rDNA transcription, rRNA processing and shuttling of pre-ribosomal subunits from nucleus to the cytoplasm. Given its substantial role in ribosome biogenesis, we sought to identify novel mechanisms by which ARF regulates ribosome biogenesis. Particularly given the high incidence of CDKN2A locus deletion and enhancement of ribosome biogenesis pathway reported in multiple cancers, gaining a thorough understanding of how ARF regulates ribosome biogenesis can help us identify novel therapeutic targets for cancer treatment. Here, we report that CRISPR-mediated disruption of ARF in human mammary epithelial cells leads to significant alterations in the nucleolar architecture and number. We further show that in addition to being part of multi-protein complexes containing Drosha and other proteins known to be involved in ribosome biogenesis, ARF immunoprecipitates with Drosha in an RNA-independent manner. Loss of ARF also leads to an overall increase in Drosha protein expression in addition to a significant redistribution of Drosha to the nucleolar compartment and enhanced association of Drosha with select rRNA precursors. Together, these results support a role for ARF in negatively regulating Drosha's rRNA processing functions.

2.2 INTRODUCTION

Ribosome biogenesis functions as a limiting factor in cell proliferation, as proliferation requires a tremendous amount of protein synthesis, a feat impossible without sufficient availability of the protein-synthesis machinery—the ribosome (1). Given its important role in cell proliferation, a strong feed-back loop exists between ribosome biogenesis and cell cycle regulators such as p53 and Rb; aberrations in ribosome biogenesis have the ability to prevent cell cycle progression through activation of the p53 and Rb tumor suppressors. P53 and Rb are further capable of feeding back into the ribosome biogenesis pathway in an inhibitory manner (2-5). It has long since been noted that highly proliferative cancer cells tend to have an increased number of nucleoli—the sites of active rDNA transcription—indicative of increased ribosome biogenesis (6). In fact, multiple oncogenes facilitate accelerated proliferation rates through upregulation of ribosome biogenesis at multiple levels (7-11) and haploinsufficiency of components required for this process have been shown to suppress tumorigenic potential of oncogenes (12). Inhibiting ribosome biogenesis pathway therefore has the potential to severely impair cancer cell proliferation. Interestingly, in depth analysis of the mode of action of multiple chemotherapeutic agents assumed to prevent cancer cell proliferation through DNA-damage inducing mechanisms have instead been shown to predominantly function through inhibition of the ribosome biogenesis pathway (13). Furthermore, it has been shown that DNA damage dependent activation of the p53 tumor suppressor only occurs in presence of nucleolar DNA damage, but not in presence of nuclear DNA damage, suggesting an important role for nucleolar compartment in surveillance of DNA integrity and inhibition of aberrant cell proliferation (14). Therefore, understanding the mechanisms regulating ribosome biogenesis and how these mechanisms are altered by deregulated oncogene or tumor suppressor

expression can significantly help us in identifying weak-links within the pathway that can be exploited for cancer treatment.

While several oncogenes have garnered a prominent role for themselves in enhancing ribosome biogenesis, the p14ARF (p19ARF in mouse) tumor suppressor has been shown to have potent inhibitory functions in the said pathway (15,16). The human p14ARF is one of two tumor suppressor proteins encoded by the INK4a/ARF (or CDKN2A) locus, the other tumor suppressor being p16INK4a. The mRNAs of the two proteins have distinct first exons, however, they splice into the same second exon in an alternative reading frame, leading to translated proteins with distinct amino acid sequences (17). Both p16INK4a and p14ARF negatively regulate cell cycle progression through Rb and p53 respectively (18). P14ARF is capable of further impeding cell proliferation through inhibition of the ribosome biogenesis pathway. Under normal cellular conditions, cells maintain very low levels of p14ARF due to its growth suppressive roles. However, hypreproliferative signals, as triggered by expression of oncogenes, strongly induce p14ARF as a protective barrier against aberrant cell proliferation (19-21). In response to such hyperproliferative signals, p14ARF can suppress cell proliferation through p53-dependent and –independent mechanisms. P14ARF is primarily a nucleolar protein and is capable of sequestering HDM2 (MDM2 in mouse), the E3 ubiquitin ligase responsible for targeting p53 for degradation. Upon its activation, p14ARF binds HDM2 and sequesters it to the nucleolus, thereby lifting its negative regulation over p53, and inducing cell cycle arrest in a p53-dependent manner (22). Additionally, p14ARF is capable of inhibition cell proliferation in a p53-independent manner, mainly through its regulatory roles in the ribosome biogenesis pathway (23,24). Particularly, p14ARF is capable of sequestering nucleolar proteins involved in rDNA transcription, rRNA processing as well as shuttling pre-ribosomal complexes from nucleus to the cytoplasm (23,25-27). In addition to its

inhibitory role in ribosome biogenesis, p14ARF has also been shown to inhibit protein translation through regulation of translation initiation factors, although the exact mode of this regulation is not yet clear.

As a negative regulator of ribosome biogenesis and protein translation, p14ARF status can have strong implication for tumor progression. As such, suppression or deletion of the CDKN2A locus is a common occurrence in multiple cancers (28-36). Identifying ARF-dependent mechanisms regulating ribosome biogenesis and their contribution toward cellular transformation allows us to gain a better understanding of the cellular processes contributing toward tumor progression in cancers with functionally deficient CDKN2A locus. Of further significance, it has been suggested that in absence of functional p53, tumor suppressive functions of ARF can have particular importance in preventing cellular transformation (33,37). As loss of p53 function is one of the most common occurrences in multiple cancers, identifying p53-independent mechanisms of ARF's cellular functions can have significant relevance for p53-deficient tumor cells.

In this study, we report a novel role for ARF in regulation of the subcellular localization and nucleolar functions of the RNase III enzyme, Drosha. We report that p14ARF interacts with Drosha and other known ribosome biogenesis components to limit rRNA processing. Loss of ARF through CRISPR-mediated gene editing, leads to an accumulation of Drosha in the nucleolus and significant gains in rRNA processing. Finally, we show that ARF loss increases Drosha's association with pre-rRNA species, making Drosha a new potential target for ARF-dependent regulation of nucleolar functions.

2.3 MATERIALS AND METHODS

Cell Culture

Human mammary epithelial cells HME-hTERT (hTERT-HME1, CRL-4010) and MCF10A were obtained from ATCC and cultured in Mammary Life Complete Medium (Lifeline Cell Technology). TLHMECs, human mammary epithelial cells immortalized with large T-antigen and telomerase, were a gift from Dr. Steven Elledge (Harvard University, Boston, MA).

CRISPR Mediated Genome Editing

Three CRISPR-cas9-GFP expression plasmids containing guide RNA targeting Exon 1-beta of *CDKN2A* locus, 5'-CCCTCCGGATTCGGCGCGCGTG-3' (HS0000505936), 5'-TTTCGTGGTTCACATCCCGCGG-3' (HS0000505943) and 5'-CCCTCGTGCTGATGCTA CTGAG-3' (HS0000505948) were obtained from Sigma-Aldrich. These plasmids were transduced into TLHMECs and sorted for cells with high GFP expression 48 hours post transduction. Single colonies were then isolated from the sorted cells and analyzed for successful p14-ARF knockout.

Plasmids and Viral Production

pLKO.1-puro constructs obtained from the Genome Institute at Washington University were used for shRNA-mediated knockdown of p14ARF. Sequences for shRNAs are 5'-GCGCTGCCCAACGCACCGAAT-3' for shARF; 5'-GCCGTCGTATTACAACGTCGT-3' for shLacZ control; and 5'-TCACAGAATCGTCGTATGCAG-3' for shLuciferase control. Lentiviral Flag-Drosha overexpression constructs were generated by PCR amplifying Drosha ORF from pCDNA4/TO/cmycDrosha (addgene 10828) using 5'-GCGATCACTAGTCCACCATGGACTACAAGGACGACGATGACAAGGGAGGA

AGTATGATGCAGGGAAACACATGTCAC-3' forward and 5'-GCGATCGCGGCCGCTTA TTTCTTGATGTCTTCAGTCTCATCTGG-3' reverse primers. The PCR product was then cloned into TOPO pCR2.1 vector for sequencing. The TOPO vectors were then digested using SpeI and NotI restriction enzymes and ligated into pLVX-IRES-Hygro (Clontech) lentiviral backbone. For production of lentivirus encoding shRNA and overexpression constructs, 293T cells were co-transfected with pCMV-VSV-G, pHR8.2ΔR and pLKO.1-puro or pLVX-Hygro constructs using Lipofectamine 2000 (Invitrogen). Viral supernatant was collected 48 hours post transfection and aliquots were frozen at -80^{0}C.

Proliferation and Foci Formation Assays

For proliferation assay, 5.0x10^4 cells per condition were plated in triplicate on day 0. Cells were harvested and counted using hemacytometer on consecutive days starting 48 hours post plating. For Foci assay, 3.0x10^3 cells per condition were plated in triplicate and cultured for 15 days. Colonies were fixed with 100% methanol and stained for 1 hour with 25% Giemsa.

Immunoblotting and Immunoprecipitation

The following antibodies were used for immunoblot analysis: rabbit anti-Drosha (Abcam, ab12286), rabbit anti-DGCR8 (Abcam, ab36865), rabbit anti-DDX5 (Bethyl, A300-523A), rabbit anti-p14ARF (Bethyl, A300-342A), mouse anti-NPM (Invitrogen, 32-5200), rabbit anti-GAPDH (Bethyl, A300-641A). For immunoprecipitation, 500 micrograms of whole cell lysate (or 1mL fraction from gel filtration assay) was incubated with rabbit anti-Drosha (Abcam, ab12286) or rabbit IgG. Protein complexes were then immunoprecipitated using Protein A Dynabeads (Life Technologies, 10002D) and eluted in SDS loading buffer.

Immunofluorescence and Confocal Microscopy

For subcellular localization of Drosha in TLHMECs and CR-p14 cell lines, cells were fixed with 10% Methanol in 10% Formalin for 15 minutes and permeabilized with 0.3% Triton X-100 in PBS for 7 minutes. Cells were blocked in 5% FBS in PBS and probed with mouse anti-NPM (Invitrogen) and rabbit anti-Drosha (Abcam). Fluorescein isothiocyanate (FITC)- and Rhodamine-X (RhoX)–conjugated secondary antibodies (Jackson ImmunoResearch) were used for visualizing NPM and Drosha. Samples were mounted with Vectashield containing DAPI (Vector Laboratories). Images were acquired with Zeiss LSM 5 PASCAL Vario Two RGB system coupled to a Zeiss Axiovert 200 microscope using a 40x water immersion lens. For subcellular localization of Drosha in TLHMECs overexpressing Flag-Drosha, cells were fixed, permeabilized and blocked as mentioned above. Cells were then probed with rabbit anti-NPM (Santa Cruz) and mouse anti-Flag (Sigma). FITC- and RhoX–conjugated secondary antibodies were used for visualizing NPM and Flag. Samples were mounted with Vectashield containing DAPI. Images were acquired with Nikon A1Rsi scanning confocal microscope using a 40x oil immersion lens.

Sub-cellular Fractionation

1-2x10^8 cells were used for isolation of nucleoli. Nuclei were collected by lysing and dounce homogenizing cells in 10mM HEPES-KOH buffer supplemented with 1.5M $MgCl_2$, 10mM KCl and 0.5mM DTT. Isolated nuclei were then sonicated using 550 Sonic Dismembrator (Fisher Scientific) and nucleoli separated through differential centrifugation over sucrose.(38) Separated fractions were then analyzed for protein expression using immunoblot analysis.

Gel Filtration Chromatography

Cells were lysed in Tween20 Lysis buffer.(39) 600 micrograms of protein was loaded onto HiPrep Sephacryl S-300 HR column (GE Healthcare Life Sciences) and protein complexes were allowed to separate based on size under constant flow and low pressure.(38) Proteins were precipitated from 1 mL fractions using 10% trichloroacetic acid (Sigma) and analyzed by immunoblotting.

Ribosomal RNA Processing

Equal numbers of TLHMEC and CR-p14 cells were grown in methionine-free DMEM containing 10% dialyzed FBS for 15 minutes. Cells were then treated with 50μCi/mL [methyl-^{3}H]-methionine and incubated at 37^{0}C for 5 minutes and chased with complete media supplemented with 10μmol/L cold methionine for indicated times. RNA was extracted from the samples using RNA-Solv Reagent (Omega Biotek R6830-02), resolved on a 1% agarose-formaldehyde gel and transferred to Amersham Hybond-XL membrane (GE Healthcare, RPN303S). RNA was then cross-linked to the membrane and sprayed with EN^{3}HANCE (PerkinElmer) before autoradiography.

Ribosomal RNA Immunopreicipitation

TLHMEC and CR-p14 cells were grown in methionine-free DMEM containing 10% dialyzed FBS for 15 minutes. Cells were then treated with 50μCi/mL [methyl-^{3}H]-methionine and incubated at 37^{0}C for 30 minutes and cells were lysed in EBC Lysis buffer (25 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40 with PMSF and protease inhibitors). Equal volumes of lysate from each cell line was incubated with Rabbit IgG (Invitrogen) or Rabbit α-Drosha (Abcam) antibodies for 2 hours at 4^{0}C. Immunoprecipitated complexes were then incubated with Protein A

Dynabeads (Life Technologies) for 15 minutes at room temperature and radiolabeled RNA from immunoprecipitation reactions was isolated using RNA Solv. For Input samples, equal volumes of the protein lysates were used for RNA extraction. Isolated RNA was resolved on 1% agarose-formaldehyde gel and transferred to Amersham Hybond-XL membrane (GE Healthcare). RNA was then cross-linked to the membrane and sprayed with EN^{3}HANCE (PerkinElmer) before autoradiography.

2.4 RESULTS

p14ARF loss alters nucleolar morphology and leads to aberrant rRNA processing

The nucleolar p14/p19ARF tumor suppressor is a potent inhibitor of ribosome biogenesis at multiple steps, including rDNA transcription, rRNA processing, and nuclear export of ribosomes (23,25-27,38). Many of the loss-of-function studies for ARF have relied on mouse cells, where a bi-allelic genetic *ARF* knockout renders cells immortal and sensitive to a single oncogenic transformation event (eg. Ras) (40,41). Similar studies have not been performed in primary human cells. We generated the first CRISPR-mediated *ARF* knock out in human mammary epithelial cells immortalized with large T antigen and hTERT (TLHMEC) by targeting unique sequences in the proximal region (within the first 20 bases) of exon 1-beta of *ARF*. This targeting method resulted in a small genomic deletion that rendered the cells deficient for p14ARF expression (Figure 2.1A). This CRISPR p14ARF cell line is referred to as CR-p14. To test if loss of human p14ARF physically altered nucleolar structure and function, we stained nucleoli using an antibody recognizing the NPM nucleolar protein. Comparison of nucleolar shape and size between ARF-proficient TLHMEC and ARF-deficient CR-p14 cell lines revealed irregular nucleolar structure (Figure 2.1B). Furthermore, an overall increase in nucleolar area was observed in the CR-p14 cells (Figure 2.1C, left panel) similar to that observed in primary mouse cells (42), with significantly more CR-p14 cells showing greater than four nucleolar foci per cells (Figure 2.1C, right panel). To assess if the observed alterations in the nucleolar architecture in CR-p14 cells resulted in altered nucleolar function, we analyzed rRNA processing between TLHMECs and CR-p14 cell lines. Newly synthesized 47S rRNA precursors were labeled with [methyl-3H]-methionine and subsequent rRNA processing was monitored over a 90-minute cold methionine chase period. The CR-p14 cell line exhibited a 3-fold higher level of newly synthesized and [methyl-3H]-

methionine-labeled 47S rRNA at t=*0* compared to parental TLHMECs (Figure 2.1D, left and right panels). Overall, the CR-p14 cells showed a 4-fold increase in the rate of 47s rRNA processing when compared to parental cells (Figure 2.1D right panel). After a 90-minute chase, the CR-p14 cells displayed significantly more processed 28S and 18S rRNAs than the parental control cells (Figure 2.1E). Together, these results are consistent with a role of p14ARF in maintaining nucleolar structure while limiting rRNA processing.

ARF and Drosha interact with proteins involved in ribosome biogenesis in an RNA-independent manner

Given the changes observed in nucleolar structure and rRNA processing upon p14ARF loss, we sought to identify unique components of the p14ARF complex, in particular, those that might explain the increased rRNA processing. Others and we had previously shown that ARF physically regulates the nucleolar-localization and activity of several proteins involved in ribosome biogenesis (16,23,24,26). We first examined whether any of these proteins were in complexes with endogenous p14ARF and how these complexes might be altered upon p14ARF loss. Whole cell lysates of TLHMECs and CR-p14 cell lines were subjected to gel filtration chromatography and protein complexes were separated based on their aggregate molecular weight. Immunoblot analysis of isolated fractions from the gel filtration eluent showed that endogenous p14ARF resided in an extremely high molecular weight complex of approximately 1,465 KDa (Figure 2.2A). In addition to p14ARF, these high molecular weight fractions also contained proteins previously identified to be involved in ribosome biogenesis, including Drosha, DDX5 and NPM (Figure 2.2A). Importantly, Drosha resided almost exclusively with p14ARF in the high molecular weight complex while DGCR8, DDX5, and NPM also appeared in lower molecular

weight fractions (Figure 2.2A). In both TLHMECs and CR-p14 cell lines, all of these proteins dramatically shifted into lower molecular weight fractions upon addition of RNase and DNase (Figure 2.2B), implying that the formation of the high 1,465 KDa fraction was dependent on RNA and/or DNA. However, a modest amount of Drosha, DDX5 and NPM remained in the large molecular weight fraction from CR-p14 cells in the presence of RNAse and DNase (Figure 2.2B), suggesting that in absence of ARF, some of these complexes might be resistant to depletion of RNA and/or DNA.

To determine whether the proteins found in the high molecular weight fraction formed complexes, we immunoprecipitated Drosha from this fraction using antibodies recognizing Drosha. We found that DGCR8, DDX5, NPM and p14ARF readily formed complexes with Drosha from the high molecular weight fraction (Figure 2.2C). Drosha bound to DGCR8, DDX5 and NPM even in absence of ARF (Figure 2.2C) or following RNase treatment (Figure 2.2D). Given our observation that RNase and DNase treatment shifted these proteins into lower molecular weight fractions, our immunoprecipitation data suggest that the formation of these individual complexes does not require RNA. However, the formation of higher order complexes in cells does appear to require RNA and/or DNA. Regardless, these results indicate that Drosha forms endogenous complexes with known regulators of nucleolar rRNA processing.

ARF regulates nucleolar localization and functions of Drosha

As ARF has been shown to sequester proteins involved in ribosome biogenesis (23,25-27), we next explored p14ARF's ability to alter Drosha's subcellular localization. We first assessed subcellular distribution of Drosha and p14ARF in two different mammary epithelial cell lines, MCF10A and TLHMEC, using biochemical fractionation of whole nuclei into nucleoplasm and

intact nucleoli (23). We found that in both cell lines, Drosha and its binding partner DGCR8 were present in both the nucleoplasm and the nucleolus, while p14ARF was found predominantly in the nucleolus (Figure 2.3A). Notably, we observed that in the MCF10A cells, which contain a genomic deletion of the *CDKN2A* locus, nucleolar levels of Drosha and DGCR8 were approximately 2-fold higher than in the nucleoplasmic compartment when compared to p14ARF-proficient TLHMECs (Figure 2.3A). To determine whether this difference in subcellular distribution of Drosha was p14ARF-dependent, we performed biochemical fractionation on parental TLHMECs and CR-p14 cells, which possess the same genetic background except for ARF status. Similar to our observation with the MCF10A cell line, we found that in p14ARF-deficient CR-p14 cells, nucleolar Drosha levels were approximately 1.6 fold greater than in the nucleoplasmic compartment (Figure 2.3B) when compared to the parental TLHMECs. However, DGCR8 distribution between the nucleolar and nucleoplasmic compartments was not significantly altered upon ARF loss. To confirm our biochemical results, we assessed the co-localization of Drosha and nucleolar NPM in TLHMEC and CR-p14 cell lines using confocal microscopy. CR-p14 cells displayed significant increases in nucleolar Drosha when compared to parental TLHMECs (Figure 2.3C), strongly implying a role for p14ARF in limiting Drosha's ability to localize to the nucleolus.

We and others have previously reported that loss of Drosha impairs pre-rRNA processing (43,44). Given the increased nucleolar expression of Drosha upon ARF loss, we wanted to analyze the functional relevance of the altered Drosha localization in nucleolar compartment. We therefore labeled TLHMECs and CR-p14 cells with [methyl-3H]-methionine and immunoprecipitated Drosha-bound RNA by incubating the total cellular protein lysate with antibody specific for Drosha. We then isolated radiolabeled RNA from the immunoprecipitation reaction, resolved them on agarose gel, transferred them to a membrane and analyzed them through autoradiography. We

once again found CR-p14 cells to have more rRNA than the control TLHMEC cells (Figure 2.3E, Input lanes). Furthermore, we found that loss of ARF enhanced association of select pre-rRNA with Drosha (Figure 2.3E, right panel, asterisk) as evident by increased amounts of immunoprecipitated rRNA in ARF-deficient cells when compared to control cells. These results suggest that ARF-dependent regulation of Drosha's nucleolar localization functions in inhibiting Drosha's association with rRNA precursors and possibly processing of said precursors.

ARF inversely regulates Drosha protein expression

Our immunofluorescence analysis of CR-p14 cells showed that in addition to an increased localization of Drosha in the nucleolar compartment, there was an apparent increase in total Drosha expression upon p14ARF loss (Figure 2.3C). To examine any possible dynamic regulation of Drosha by p14ARF, we used immunoblot analysis to compare Drosha protein levels in several different contexts of p14ARF expression. In addition to the CR-p14 CRISPR deleted p14ARF cell line, we also used shRNAs targeting exon 1-beta of endogenous p14ARF transcripts in both HME-hTERT and TLHR (TLHMECs expressing Ha-Ras) cells. In all conditions of p14ARF loss, we found an approximately 2- to 4-fold increase in Drosha protein expression when compared to control cells (Figure 2.4A, 2.4B). Conversely, overexpression of p14ARF in MCF10A or in TLHMEC cells, showed a significant decrease in Drosha protein expression when compared to the control cells (Figure 2.4C). Taken together, our data point to a prominent role for p14ARF in inhibiting Drosha's nucleolar functions through negative regulation of Drosha expression and nucleolar localization.

2.5 DISCUSSION

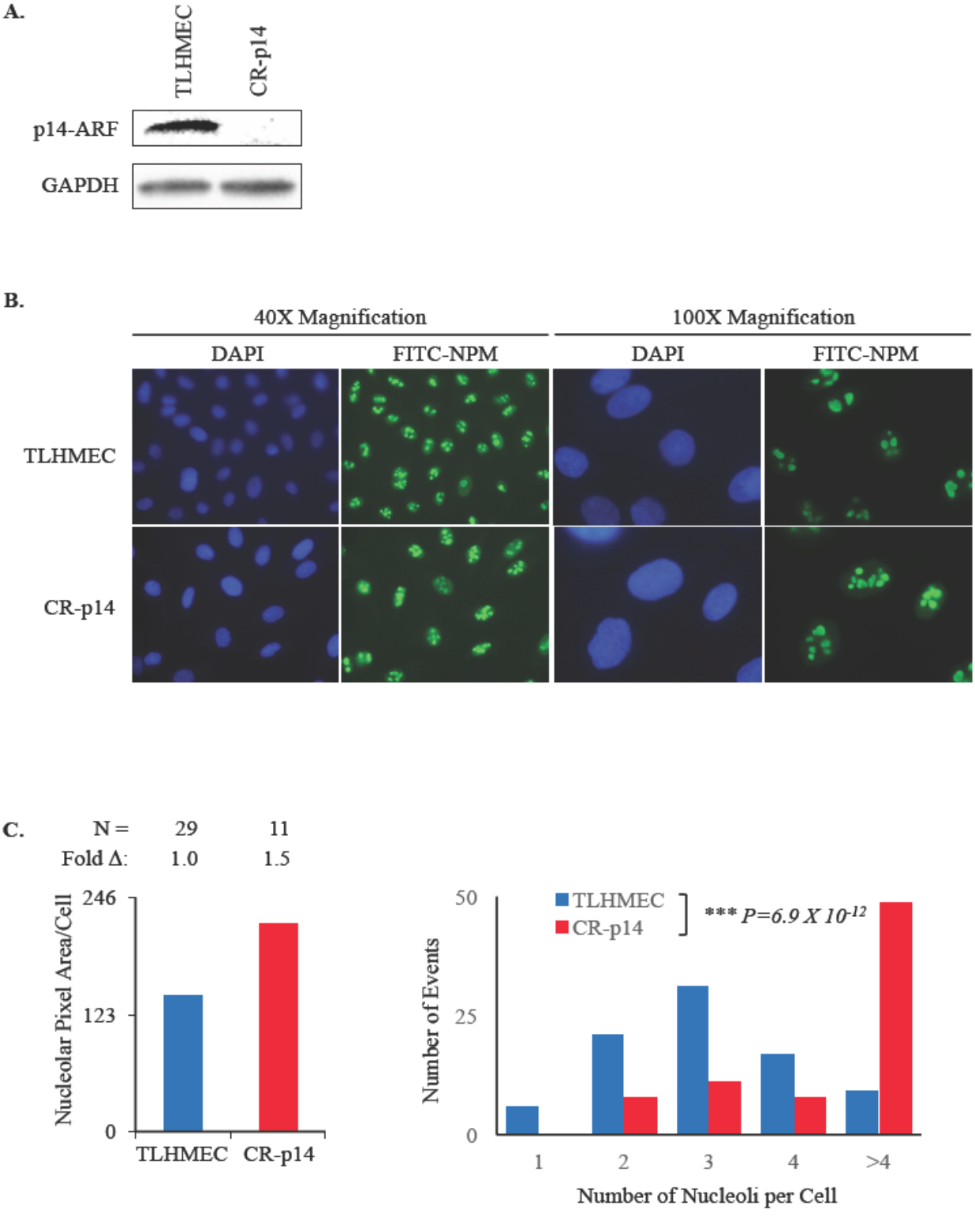
Since its discovery in 1995, the tumor suppressive role of ARF has been expanding, starting with the p53-dependent inhibition of cell cycle progression to p53-independent inhibition of ribosome biogenesis (17,23,24,26,42,45-47). Upregulation of the ribosome biogenesis pathway has been shown to be a common feature of cancer cells, the upregulation of ribosome production being a necessary means to meet the demand for increased protein synthesis required by enhanced cell proliferation rates. Loss of the CDKN2A locus encoding the p14ARF tumor suppressor is a frequent event in cancers, with a strong correlation toward poor prognosis in many cases (29-31,33,36,48,49). In addition to activation of oncogenes that can increase ribosome production, loss of functional ARF could be another mechanism by which the cancer cells ensure increased ribosome production, given the strong inhibitory role ARF plays in ribosome biogenesis. We therefore sought to identify novel mechanisms by which ARF regulates the ribosome biogenesis pathway, so as to gain a better understanding of how ARF loss could contribute to tumorigenesis.

We generated the first p14ARF-specific human knockout cell line using single guide RNAs targeting sequences unique to human ARF exon 1-beta with the CRISPR-Cas9 genome editing system. We showed that these human p14ARF knockout cells harbored tremendous gains in both nucleolar size and number, a hallmark for increased ribosome biogenesis, and rRNA processing rates. Moreover, these human ARF knockout cells had increased localization of Drosha to the nucleolus, presumably in an effort to significantly accelerate the rRNA production that we had observed.

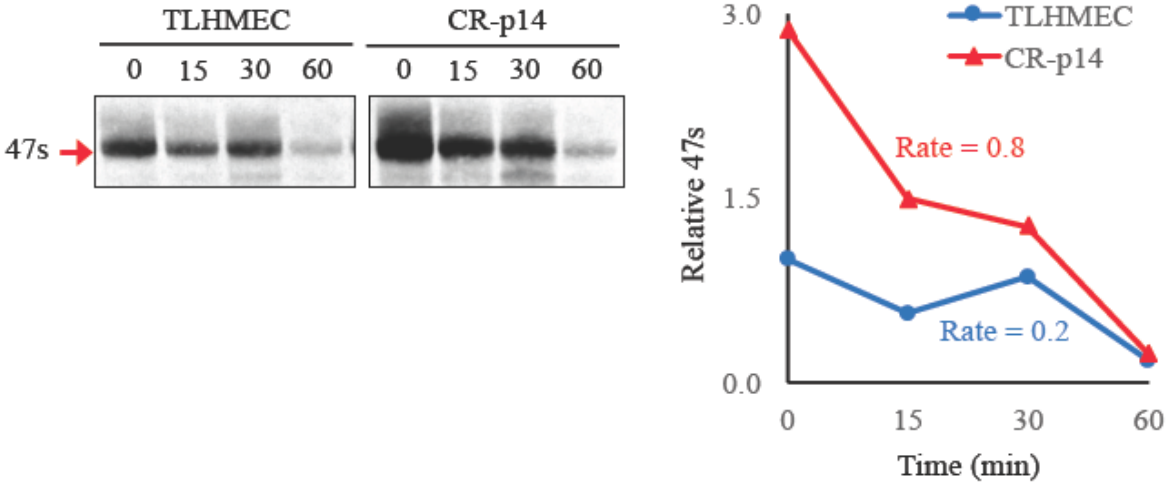
We had previously reported that the mouse p19ARF tumor suppressor was able to regulate Drosha mRNA translation to suppress Drosha protein expression (44), providing a possible connection between ARF, Drosha, and nucleolar functions. We have now greatly expanded on this

first observation. Human mammary epithelial cells exhibited a large complex or complexes containing Drosha, DGCR8, ARF, DDX5, and NPM that formed in the presence and absence of RNA/DNA. These complexes were at least 1,465 KDa large, but it is as yet unclear whether this is a single large Drosha complex or several distinct large Drosha complexes. Nonetheless, Drosha readily precipitated with DGCR8, DDX5, and NPM in the presence and absence of p14ARF, indicating that p14ARF is not necessary for the formation of these complexes. However, we cannot rule out the possibility that p14ARF regulates the subcellular localization or activity of Drosha-containing complexes. Notably, p19ARF prevents the nucleolar accumulation of DDX5 (23) and sequesters both MDM2 (47) and NPM (26) in the nucleolus, lending precedent to ARF's altering of protein localization as a means of regulation. As such, we found significant accumulation of Drosha in the nucleolus of p14ARF-deficient human cells, echoing a mechanism employed by DDX5 in its regulation of rRNA transcription in the absence of p19ARF (23). Furthermore, immunoprecipitation of Drosha-associated RNA in TLHMECs and CR-p14 showed increased association of select pre-rRNA transcripts with Drosha in absence of ARF. These results suggest that ARF-dependent regulation of Drosha's nucleolar localization could potentially function in inhibiting rRNA processing. Previous studies have shown that Drosha and DDX5 form complexes, underscoring the potential for these two proteins to work in concert in the absence of ARF to drive nucleolar processes (50). Further interrogation of the nucleolar Drosha rRNA processing complexes could reveal how ribosome biogenesis responds to the growing needs of tumor cells.

2.6 FIGURES


A.
TLHMEC
CR-p14
p14-ARF
GAPDH
B.
40X Magnification
100X Magnification
DAPI
FITC-NPM
DAPI
FITC-NPM
TLHMEC
CR-p14
C.
N =
29
11
Fold Δ:
1.0
1.5
Nucleolar Pixel Area/Cell
246
123
0
TLHMEC
CR-p14
TLHMEC
CR-p14
*** P=6.9 X 10-12
Number of Events
50
25
0
1
2
3
4
>4
Number of Nucleoli per Cell

D.


TLHMEC
CR-p14
0 15 30 60 0 15 30 60
47s
3.0
1.5
0.0
Relative 47s
TLHMEC
CR-p14
Rate = 0.8
Rate = 0.2
0 15 30 60
Time (min)

E.

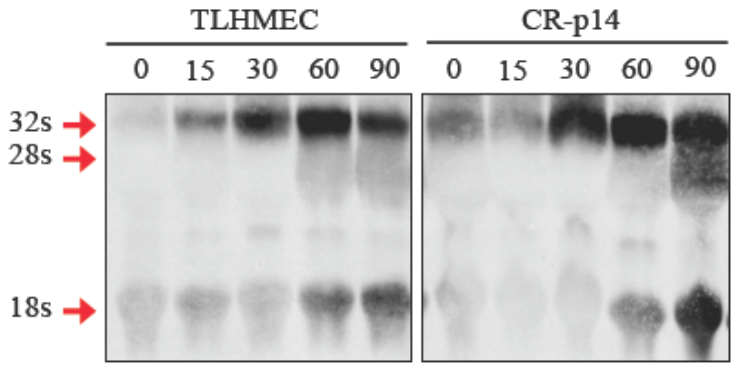
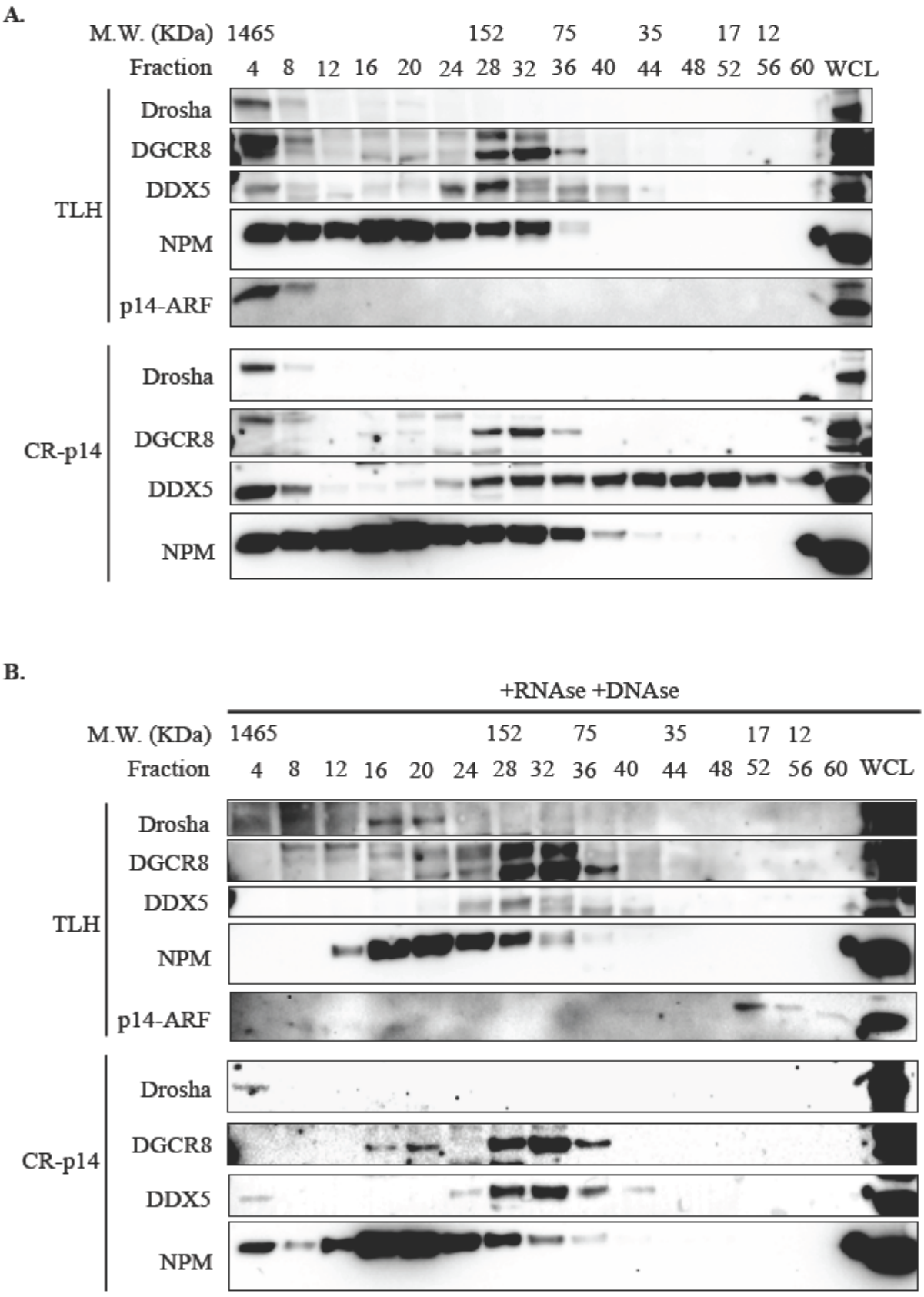
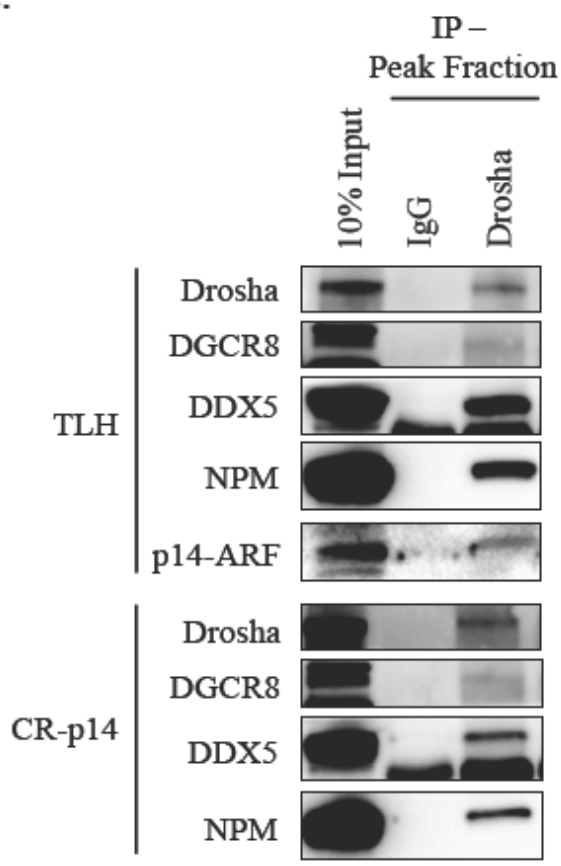

TLHMEC
CR-p14
0 15 30 60 90 0 15 30 60 90
32s
28s
18s

Figure 2.1 – ARF loss alters nucleolar morphology and functions. A. Immunoblot analysis to confirm CRISPR-mediated knockout of p14ARF in TLHMECs. Cells of the indicated genotype were lysed and separated proteins were immunoblotted for the designated proteins. **B.** Immunofluorescence analysis of nucleolar structure using nucleolar marker Nucleophosmin (NPM, green) in TLHMECs and CR-p14 TLHMECs. The indicated cells were fixed, permeabilized and stained with antibodies recognizing human NPM. Nuclei were marked with DAPI stain. **C.** Quantitation of nucleolar area per cell in TLHMECs and CR-p14 TLHMEC. (left panel). Quantification of number of nucleoli per cell in TLHMECs and CR-p14 cells (right panel). **D.** TLHMECs and CR-p14 cells were labled with [methyl-3H]-methionine and chased for indicated times. Radiolabeled RNA was separated on agarose gel and visualized by autoradiography post transfer to membrane (left panel). Relative 47s band intensity is quantitated and shown in right panel. 47s band intensities were normalized to TLHMEC sample at t = 0. **E.** Representative rRNA processing events in parental and CR-p14 TLHMECs. Radiolabeled 32s, 28s and 18s rRNA processing events are visualized by autoradiography.


A.
M.W. (KDa) 1465 152 75 35 17 12
Fraction 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 WCL
TLH
Drosha
DGCR8
DDX5
NPM
p14-ARF
CR-p14
Drosha
DGCR8
DDX5
NPM
B.
+RNAse +DNAse
M.W. (KDa) 1465 152 75 35 17 12
Fraction 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 WCL
TLH
Drosha
DGCR8
DDX5
NPM
p14-ARF
CR-p14
Drosha
DGCR8
DDX5
NPM

C.


IP –
Peak Fraction
10% Input
IgG
Drosha
TLH
Drosha
DGCR8
DDX5
NPM
p14-ARF
CR-p14
Drosha
DGCR8
DDX5
NPM

D.

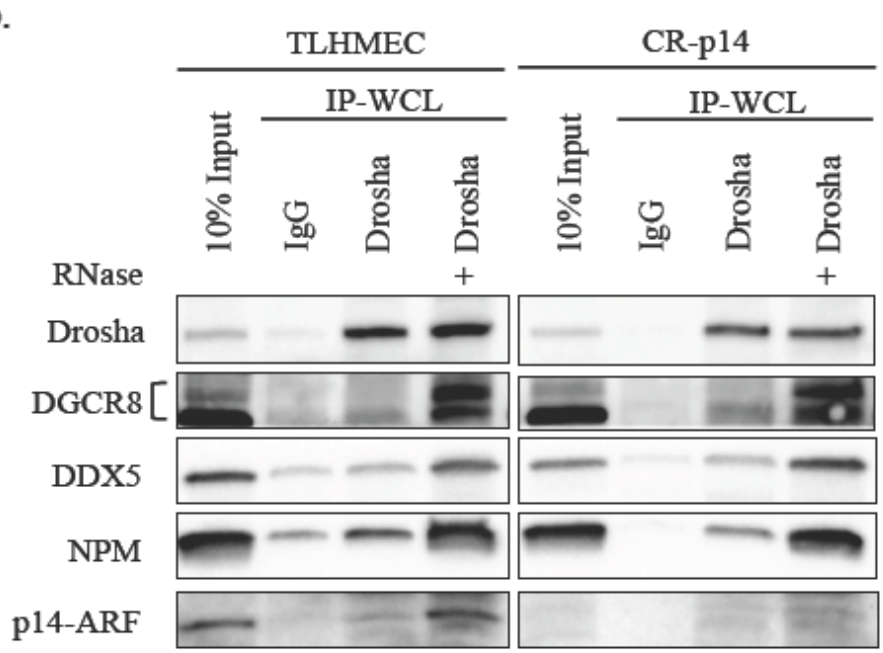
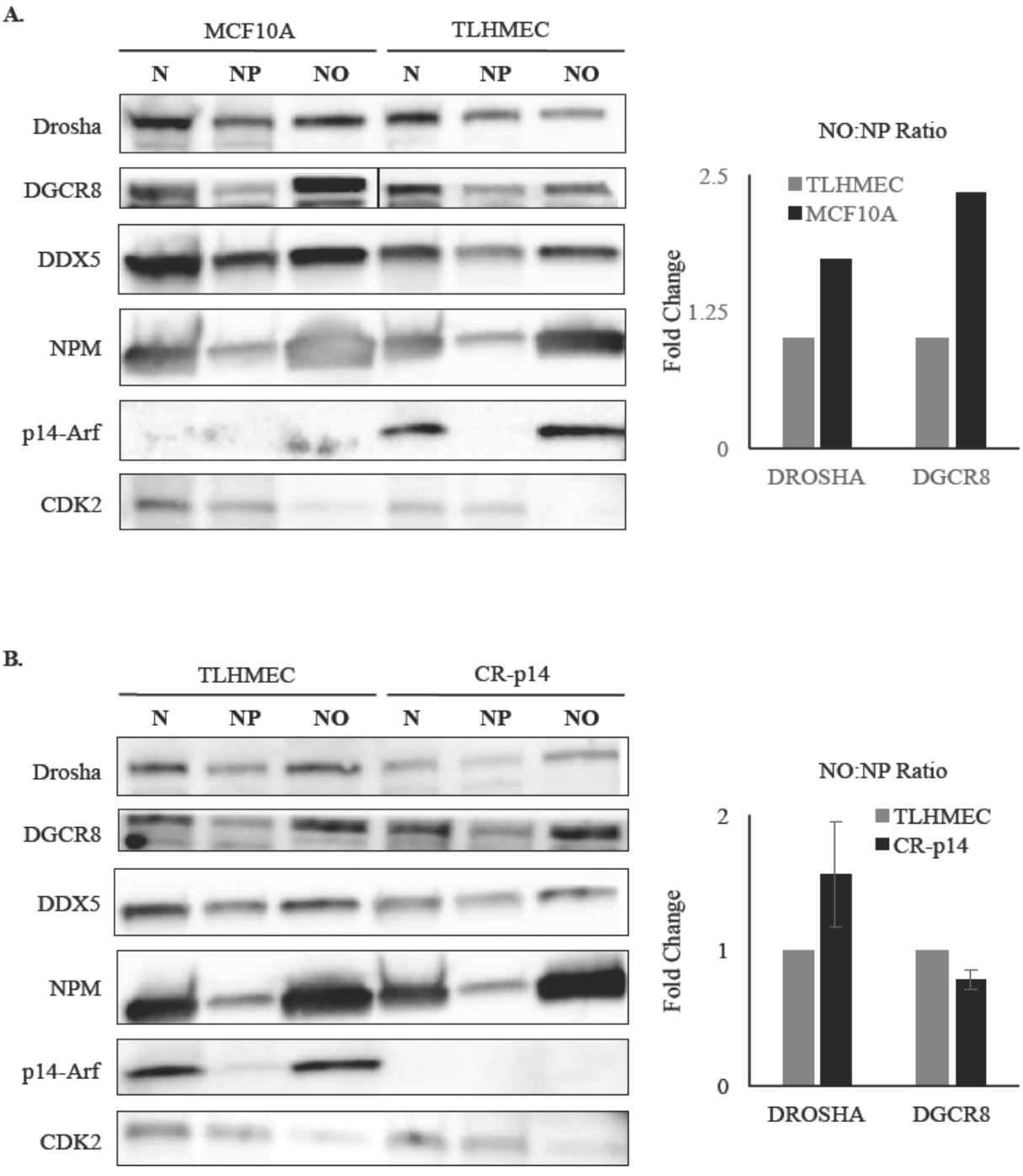
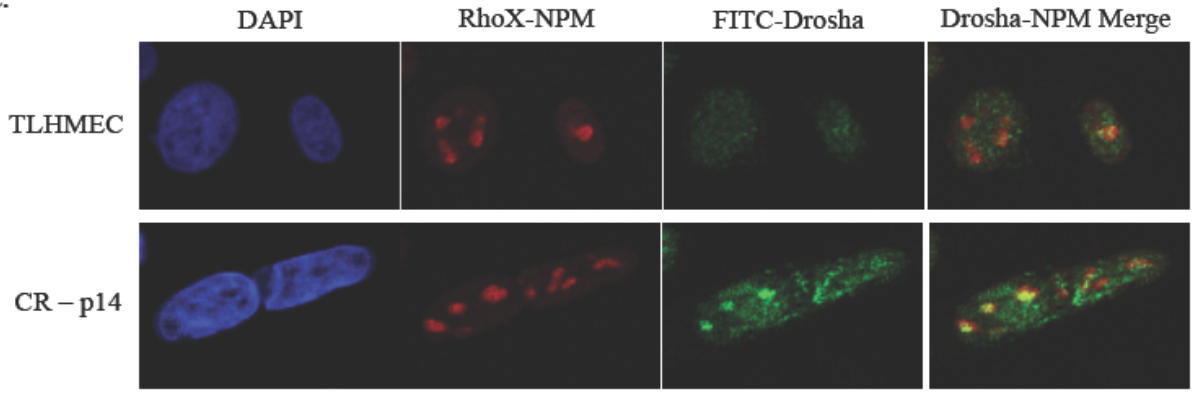

TLHMEC
CR-p14
IP-WCL
IP-WCL
10% Input
IgG
Drosha
+ Drosha
10% Input
IgG
Drosha
+ Drosha
RNase
Drosha
DGCR8
DDX5
NPM
p14-ARF

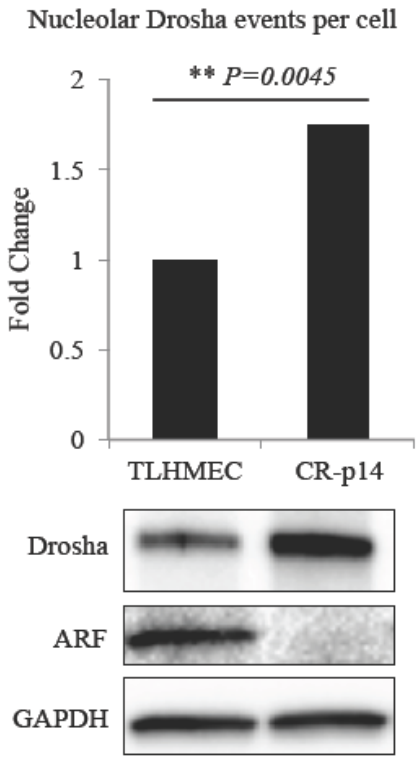
Figure 2.2 – p14ARF and Drosha interact with proteins involved in ribosome biogenesis. A. Immunoblot analysis of fractions from Sephacryl gel filtration column. Lysates from TLHMEC and CR-p14 cells were loaded onto Sephacryl gel filtration columns and eluted under identical flow rates and low pressure. Fractions were collected, proteins precipitated with trichloroacetic acid, and identified by SDS-PAGE and immunoblotting with the indicated antibodies. **B.** Immunoblot analysis of fractions from RNase A and DNase treated cell lysates. **C.** Immunoprecipitation of Drosha-interacting proteins using antibodies recognizing Drosha from peak fraction, fraction 5 of the gel filtration column from A. **D.** Immunoprecipitation of Drosha-interacting proteins from whole cell lysates mock treated or treated with 2 Units of RNase. Immune complexes were separated by SDS-PAGE and immunoblotted with the indicated antibodies.


A.
MCF10A
TLHMEC
N
NP
NO
N
NP
NO
Drosha
DGCR8
DDX5
NPM
p14-Arf
CDK2
NO:NP Ratio
TLHMEC
MCF10A
Fold Change
2.5
1.25
0
DROSHA
DGCR8
B.
TLHMEC
CR-p14
N
NP
NO
N
NP
NO
Drosha
DGCR8
DDX5
NPM
p14-Arf
CDK2
NO:NP Ratio
TLHMEC
CR-p14
Fold Change
2
1
0
DROSHA
DGCR8

C.


DAPI
RhoX-NPM
FITC-Drosha
Drosha-NPM Merge
TLHMEC
CR – p14

D.


Nucleolar Drosha events per cell
** P=0.0045
Fold Change
2
1.5
1
0.5
0
TLHMEC
CR-p14
Drosha
ARF
GAPDH

E.

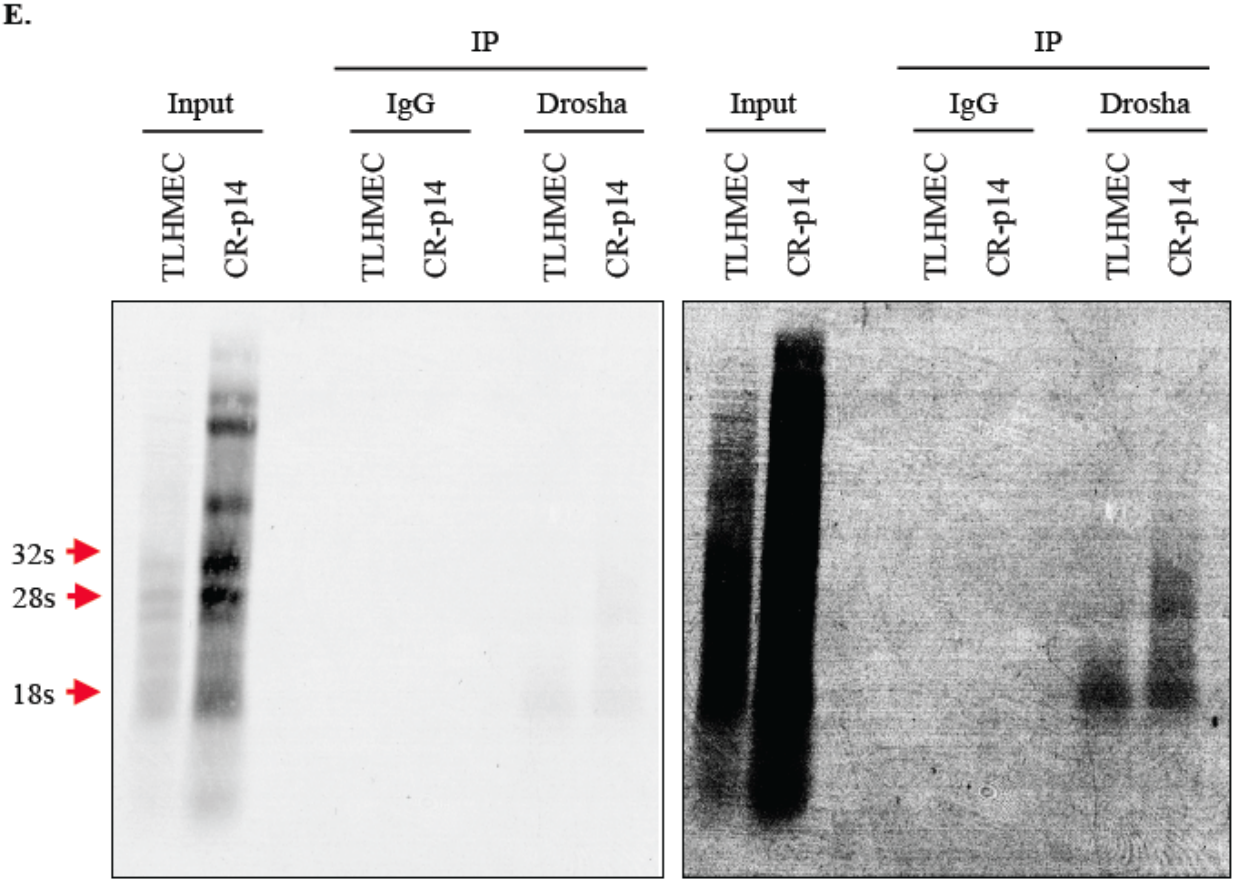
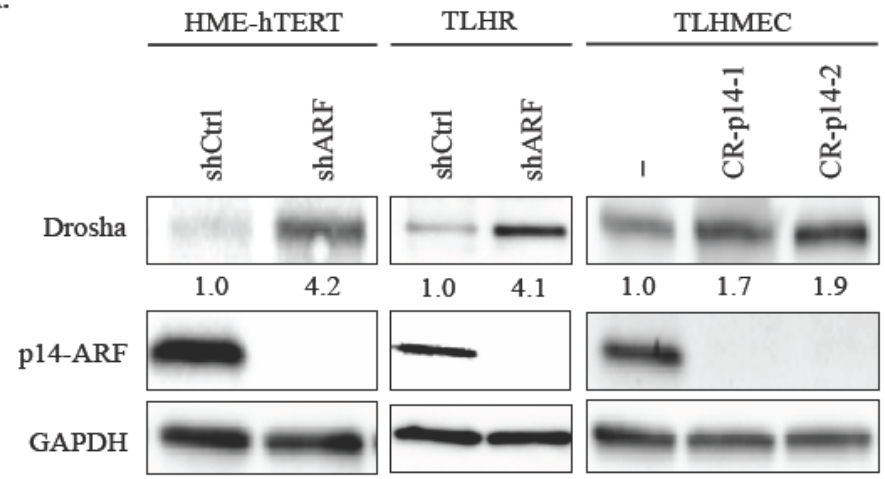
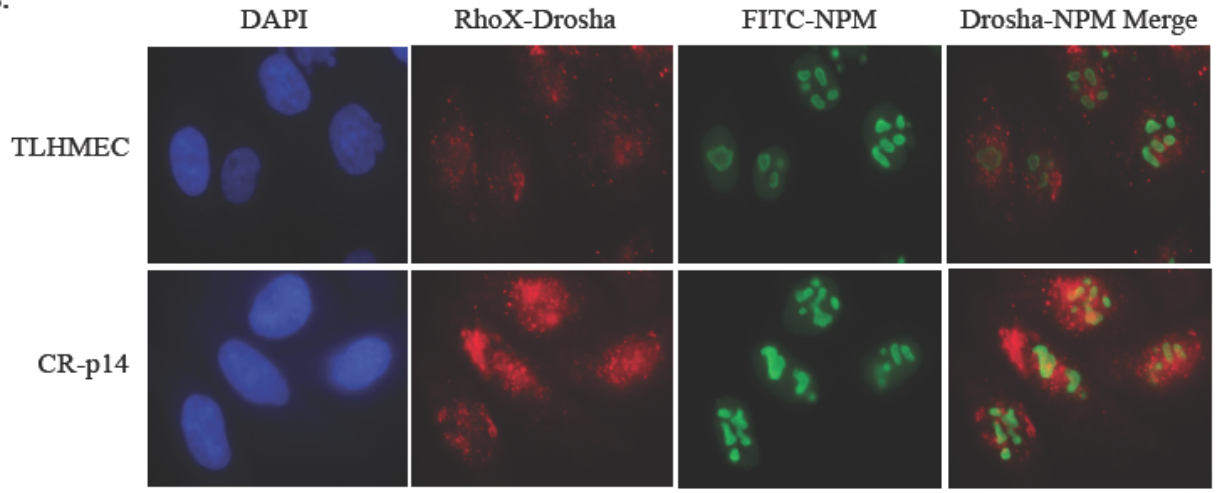

IP
IP
Input
IgG
Drosha
Input
IgG
Drosha
TLHMEC
CR-p14
TLHMEC
CR-p14
TLHMEC
CR-p14
TLHMEC
CR-p14
TLHMEC
CR-p14
TLHMEC
CR-p14
32s
28s
18s

Figure 2.3 – Nucleolar localization of Drosha increases upon p14ARF loss. A. Subcellular distribution of indicated proteins in different mammary epithelial cell lines. N = nuclear, NP = Nucleoplasm, NO = Nucleolar (left panel). Quantitation of nucleolar levels of indicated proteins relative to nucleoplasmic protein levels (right panel). **B.** Subcellular fractionation of TLHMEC and CR-p14 cells (left panel). Quantitation of nucleolar levels of indicated proteins relative to nucleoplasmic protein levels (right panel, n = 3). **C.** Confocal imaging of TLHMECs and CR-p14 cells for Drosha and NPM co-localization (left panel). **D.** Quantitation of nucleolar Drosha events per cell represented by NPM-Drosha co-localization (top right panel). Immunoblot analysis of TLHMECs and CR-p14 cells (bottom right panel). **E.** Equal volume of [methyl-3H]-methionine labeled TLHMEC and CR-p14 whole cell protein lysates were incubated with Drosha-specific antibody, or control IgG. Radiolabeled RNA was isolated from the immunoprecipitation reactions, separated on agarose gel, transferred to membrane and visualized through autoradiography.

A.


HME-hTERT
TLHR
TLHMEC
shCtrl
shARF
shCtrl
shARF
–
CR-p14-1
CR-p14-2
Drosha
1.0
4.2
1.0
4.1
1.0
1.7
1.9
p14-ARF
GAPDH

B.


DAPI
RhoX-Drosha
FITC-NPM
Drosha-NPM Merge
TLHMEC
CR-p14

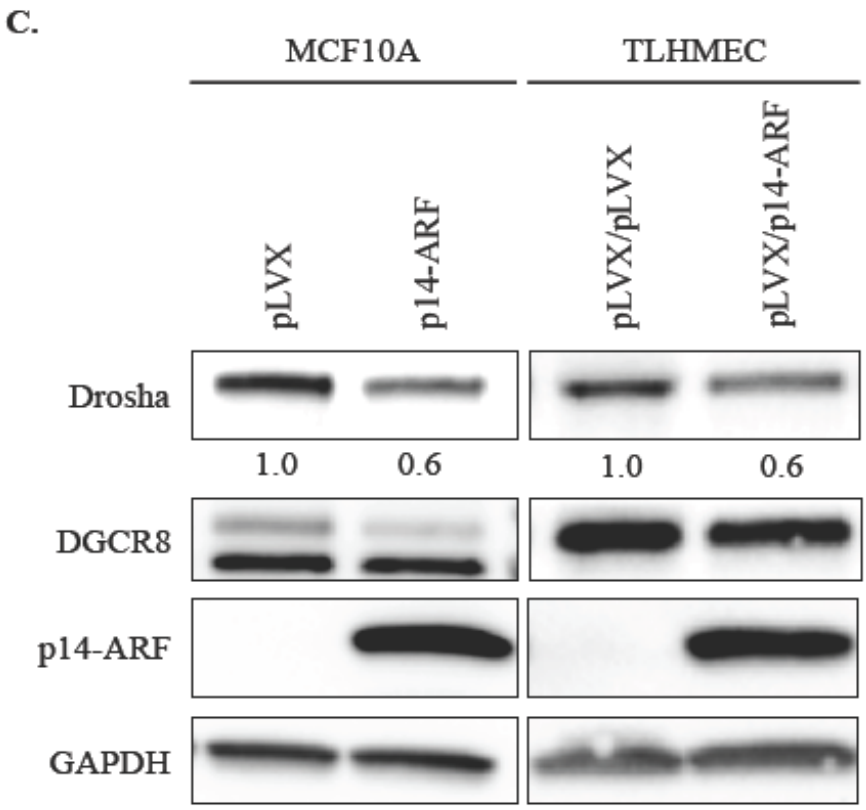


Figure 2.4 – ARF inversely regulates Drosha protein expression. A. shRNA-mediated knockdown and CRISPR mediated knockout of p14ARF. HME-hTERT and TLHR human cells were transduced with shRNAs targeting endogenous human p14ARF, lysed and immunoblotted with the indicated antibodies. Conversely, TLHMECs were transduced with single guide RNAs targeting exon 1-beta of ARF and CRISPR-Cas9 constructs. Two independent CR-p14 clones were selected, lysed, and immunoblotted with the indicated antibodies. **B.** Immunofluorescence analysis of TLHMECs and CR-p14 cell lines for Drosha protein expression. **C.** ARF overexpression in *ARF*-null MCF10A and TLHMEC cells. Each cell line was transduced with p14ARF expression constructs, lysed and immunoblotted with the indicated antibodies.

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CHAPTER 3:

DROSHA OVEREXPRESSION ENHANCES RIBOSOMAL RNA PROCESSING AND CELL PROLIFERATION PROPERTIES

3.1 ABSTRACT

Drosha is an RNAse III endonuclease capable of cutting double-stranded RNAs. It is part of the microprocessor, a multi-protein complex involved in the first step of microRNA (miRNA) processing. In addition to its role in miRNA processing, Drosha also participates in the processing of ribosomal RNA (rRNA) and destabilization of mRNAs. The ability of Drosha to regulate multiple RNA species within the cell makes it an important mediator of cellular homeostasis. Both genomic amplification and post-transcriptional overexpression of Drosha has been reported in multiple cancers, including breast cancer. Drosha expression has been shown to have prognostic value in some cancers while showing a strong correlation with disease progression in others. Unfortunately, our minimal understanding of the mechanisms regulating Drosha expression and function, as well as the functional contribution of different arms of Drosha's cellular functions toward tumorigenesis makes the design of targeted therapies for Drosha overexpressing cancers difficult. Here, we report that overexpression of Drosha enhances rRNA processing in addition to cell proliferation properties. We further provide evidence for a cross-regulatory loop between Drosha and ARF in regulation of nucleolar functions.

3.2 INTRODUCTION

Drosha is a ubiquitously expressed protein with functional significance in multiple cellular pathways. Encoded by the RNASEN gene, Drosha is an RNaseIII enzyme with specific activity against double-stranded RNA (dsRNA). Originally, Human Drosha was identified as an RNase with important role in the ribosome biogenesis pathway; anti-sense inhibition of Drosha expression leads to an accumulation of rRNA precursors (1). Since then, a few other reports have confirmed Drosha's role in rRNA processing, as a loss of Drosha expression consistently impaired rRNA processing (2,3). Although our mechanistic understanding of Drosha's functional contribution toward the ribosome biogenesis pathway is limited, alterations in Drosha expression have the potential to significantly alter gene expression through the ribosome biogenesis pathway.

Unlike its role in ribosome biogenesis pathway, Drosha's contribution to the miRNA biogenesis pathway has been extensively studied. As the nuclear RNase, with aid from its binding partner DGCR8, Drosha has been shown to be necessary for cleaving of the pri-miRNA transcripts into the pre-miRNAs (4). These pri-miRNAs are then transported out to the cytoplasm to undergo further processing events to generate the mature miRNAs, which are once again capable of regulating gene expression through inhibition of mRNA translation and protein expression.

More recently, it has been shown that in addition to its ability to impact miRNA-dependent regulation of mRNA stability and protein expression, Drosha is capable of regulating mRNA stability directly by cleaving hairpin structures in select mRNA species (5). To further emphasize the important role Drosha plays in regulating gene expression, Drosha has been implicated in transcriptional control of gene expression in an RNA-cleavage independent manner (6).

Given its central role in multiple cellular pathways regulating gene expression, it is perhaps of no surprise that alterations in Drosha expression are frequent occurrences in many cancers (7-

15). In fact, Drosha expression has been shown to have prognostic value and is capable of predicting outcomes in various cancers (16-20). Identifying the functional role Drosha plays in cancers with strong correlation between Drosha expression and survival outcomes can provide significant insight into therapeutic design for treating these cancers.

Surprisingly, Drosha has been suggested to play an oncogenic role in some cancers while suppressing tumor progression in others (7-25). To emphasize this dual nature of Drosha, unpublished data from our lab has shown that shRNA mediated knockdown of Drosha protein expression has different outcomes in different breast cancer cell lines; some cell lines were extremely sensitive to Drosha knockdown and died via the apoptotic pathway while other were insensitive to the knockdown (26). As a protein with the ability to functionally contribute to a multitude of cellular pathways regulating gene expression, the molecular basis of the apparent dual role played by Drosha in different cancers could depend on the specific Drosha-regulated pathway altered in the cancer cell. Unfortunately, the miRNA pathway has garnered the majority of attention in deciphering the potential mechanisms by which alterations in Drosha contribute toward tumorigenesis. Determining how alterations in Drosha expression impact pathways, other than the miRNA pathway, in which it functionally participates can give us a better understanding of the role Drosha plays in tumorigenesis.

In addition to delineating the functional contribution of Drosha in tumorigenesis, identifying mechanisms regulating Drosha expression and function can provide significant insight into developing targeted therapies for cancers in which Drosha has significant functional contribution. Unfortunately, other than the ability of c-MYC oncogene to positively regulate Drosha transcription (27) and the ability of DGCR8 to stabilize Drosha protein through protein-

protein interaction (28), our understanding of mechanisms regulating Drosha expression and functions is rather limited.

In our current study, we report that overexpression of Drosha acts in an oncogenic manner and enhances cell proliferation and colony formation capabilities of cells, particularly in a p53 and Rb impaired cellular background. Furthermore, we show that overexpressed Drosha localizes to the nucleolus of cells and contributes toward enhancement of rRNA synthesis and processing. Additionally, overexpression of the tumor suppressor p14ARF, which has been proven to have a strong inhibitory role in ribosome biogenesis pathway, leads to an abrogation of Drosha-mediated enhancement of cell proliferation properties. We further report a cross-regulatory loop between p14-ARF and Drosha, as a potential anti-tumorigenic barrier.

3.3 MATERIALS AND METHODS

Cell culture

Human mammary epithelial cells carrying telomerase (hTERT-HME1, CRL-4010) were purchased from ATCC and maintained in Mammary Life Complete Medium (Lifeline Cell Technology). TLHMECs, immortalizes with large T-antigen and telomerase, were a gift from Dr. Steven Elledge (Harvard University, Boston, MA).

Plasmids and viral production

Lentiviral Flag-Drosha overexpression constructs were generated by PCR amplifying Drosha ORF from pCDNA4/TO/cmycDrosha (addgene 10828) using 5'-GCGATCACTAGTCCACCATGGACTACAAGGACG ACGATGACAAGGGAGGAAGTATGATGCAGGGAAACACATGTCAC-3' forward and 5'-GCGATCGCGG CCGCTTATTTCTTGATGTCTTCAGTCTCATCTGG-3' reverse primers. The PCR product was then cloned into TOPO pCR2.1 vector for sequencing. For 9R-Drosha construct, site-directed mutagenesis of Flag-Drosha TOPO vector was performed using the Quickchange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and 5'-GACAAGGGAGGAAGTAGAAGGCGCCGACGGAGGAGGCGCAGGATGATGCAG GGAAACACA -3' primer and its reverse complement. The TOPO vectors were then digested using SpeI and NotI restriction enzymes and ligated into pLVX-IRES-Hygro (Clontech) lentiviral backbone.

For production of lentivirus encoding overexpression constructs, 293T cells were co-transfected with pCMV-VSV-G, pHR8.2ΔR and pLVX-Hygro constructs using Lipofectamine 2000

(Invitrogen). Viral supernatant was collected 48 hours post transfection and aliquots were frozen at -80^{0}C.

Proliferation and Foci formation Assays

For proliferation assay, 50,000 cells per condition were plated in triplicate on day 0. Cells were harvested and counted using hemacytometer on consecutive days starting 48 hours post plating. For Foci assay, 3,000 cells per condition were plated in triplicate and cultured for 10 days. Colonies were fixed with 100% methanol and stained for 1 hour with 25% Giemsa.

Immunoblotting and Immunoprecipitation

The following antibodies were used for western blot analysis: rabbit anti-Drosha (abcam, ab12286), rabbit anti-DGCR8 (abcam, ab36865), rabbit anti-DDX5 (Bethyl, A300-523A), rabbit anti-p14ARF (Bethyl, A300-342A), mouse anti-NPM (Invitrogen, 32-5200), rabbit anti-GAPDH (Bethyl, A300-641A).

Immunofluorescence and Confocal Microscopy

For subcellular localization of Drosha in TLHMECs and CR-p14 cell lines, cells were fixed with 10% Methanol in 10% Formalin for 15 minutes and permeablized with 0.3% TritonX-100 in PBS for 7 minutes. Cells were blocked in 5% FBS in PBS and probed with mouse anti-NPM (Invitrogen) and rabbit anti-Drosha (abcam). Fluorescein isothiocyanate (FITC)- and Rhodamine-X (RhoX)–conjugated secondary antibodies (Jackson ImmunoResearch) were used for visualizing NPM and Drosha. Samples were mounted with Vectashield containing DAPI (Vector Laboratories). Images

were acquired with Zeiss LSM 5 PASCAL Vario Two RGB system coupled to a Zeiss Axiovert 200 microscope using a 40x water immersion lens.

For subcellular localization of Drosha in TLHMECs overexpressing Flag-Drosha, cells were fixed with 10% Methanol in 10% Formalin for 15 minutes and permeablized with 0.3% TritonX-100 in PBS for 7 minutes. Cells were blocked in 5% FBS in PBS and probed with rabbit anti-NPM (Santa Cruz) and mouse anti-Flag (). FITC- and RhoX–conjugated secondary antibodies were used for visualizing NPM and Flag. Samples were mounted with Vectashield containing DAPI. Images were acquired with Nikon A1Rsi scanning confocal microscope using a 40x oil immersion lens.

Ribosomal RNA Processing

Equal number of TLHMECs and CR-p14s or TLHMEC-pLVX and TLHMEC-Drosha cells were grown in methionine-free DMEM containing 10% dialyzed FBS for 15 minutes. Cells were then treated with 50 μCi/mL [methyl-3H]-methionine and incubated at 37^0C for 5 minutes and chased with complete media supplemented with 10 μmol/L cold methionine for indicated times. RNA was extracted from the samples using RNA-Solv Reagent (Omega Biotek R6830-02), resolved on a 1% agarose-formaldehyde gel and transferred to Amersham Hybond-XL membrane (GE Healthcare, RPN303S). RNA was then cross-linked to the membrane and sprayed with EN^3HANCE (PerkinElmer) before autoradiography.

3.4 RESULTS

Drosha overexpression enhances cell proliferation and foci forming abilities of p53- and Rb-impaired cells

Overexpression of Drosha has been reported to be a frequent occurrence in multiple cancers (12-15,24). To determine how overexpression of Drosha contributes to cell proliferation properties, we generated a lentiviral Flag-epitope tagged Drosha expression construct. Expression of this Flag-Drosha construct in primary human mammary epithelial cells immortalized with hTERT (HME-hTERT) induced a robust Drosha protein expression compared to the control cells (Figure 3.1A). In order to determine if the overexpressed Drosha altered cell proliferation properties, we plated equal number of control and Drosha overexpressing HME-hTERTs and counted total number of cells on consecutive days post plating. We found that overexpression of Drosha did not significantly alter proliferation rates of cells (Figure 3.1B). To asses if Drosha overexpression could contribute to cellular transformation by means other than accelerated proliferation rates, we tested the colony formation ability of Drosha overexpressing cells. To test this, we plated cells at low density and allowed the cells to grown undisturbed for 15 days. We then stained the resultant colonies with Giemsa stain and evaluated alterations in colony number and colony size upon Drosha overexpression. We observed that the Drosha overexpressing cells formed slightly bigger colonies when compared to the control cells, with the overall area of the colonies formed by Drosha overexpressing cells being significantly greater than the control cells (Figure 3.1C). These results suggest a possible role for Drosha in enhancing long-term cell properties.

P53 and Rb are two tumor suppressor proteins with important roles in preventing immortalization of cells (29). Given our observation that Drosha overexpression enhanced

clonogenic nature of cells, we wanted to test if overexpression of Drosha in p53 and Rb deficient cells could further enhance Drosha's ability to positively regulate cell proliferation. We therefore overexpressed Drosha in human mammary epithelial cells immortalized with hTERT and expressing SV40 large T antigen, which impairs p53 and Rb functions (TLHMECs). Unlike in the primary cells, we found that overexpression of Drosha in the TLHMECs increased short-term cell proliferation (Figure 3.2B) in addition to an enhancement of colony forming ability of cells (Figure 3.2C). These observations indicate that Drosha can enhance cell proliferation properties in absence of surveillance mechanisms preventing aberrant cycling of cells.

Overexpressed Drosha protein localizes to nucleolus and enhances rRNA synthesis and processing

Alterations in both rRNA biogenesis as well as miRNA biogenesis have been shown to behave in an oncogenic manner (30-38), and Drosha with important roles in both of these pathways has the potential to enhance cell proliferation through either of these pathways. As rRNA processing and miRNA processing occur in different subcellular compartments, we used immunofluorescence labeling to determine if our Flag-Drosha construct had preferential subcellular localization. Confocal microscopic analysis of control and Drosha overexpressing TLHMECs showed Flag-Drosha to be present both in the nucleoplasm and nucleoli of Drosha overexpressing cells. Interestingly, we found many of the Drosha overexpressing cells to have an almost exclusive nucleolar signal for Flag-Drosha, visualized through co-localization of Flag and nucleolar NPM (Figure 3.3B). This observation led us to analyze the impact Drosha overexpression had on nucleolar functions.

Previously, Drosha has been implicated in rRNA processing through loss of function studies, where inhibiting Drosha expression led to an accumulation of pre-rRNA products, suggesting a necessity for Drosha in processing of pre-rRNA (1,2). However, the effect of Drosha overexpression on rRNA biogenesis is largely unknown. Therefore, to analyze if Drosha overexpression can enhance rRNA synthesis and processing, we performed a pulse-chase [methyl-^{3}H]-methionine labeling of newly synthesized rRNA and analyzed 47S rRNA processing over a 90-minute time period in TLHMECs. We found that Drosha overexpressing cells started with twice as much 47S as control cells and processed the 47S rRNA at a 2-fold higher rate than the control cells (Figure 3.4A & B). We further found a faster accumulation of the 32s, 28s and 18s rRNA products in Drosha overexpressing cells when compared to the control cells (Figure 3.4C & D). These results indicate that, in a p53 and Rb-deficient setting, Drosha is capable of enhancing rRNA synthesis and processing.

Inhibiting ribosome biogenesis negates proliferative advantage conferred by Drosha overexpression

Given the increased rRNA synthesis and processing evident in Drosha overexpressing TLHMECs, we wondered if perhaps the enhanceb nd proliferation and colony formation observed in the Drosha overexpression cells could depend on the increased nucleolar processes. Ribosome biogenesis is a tightly regulated process, aberrations in which are capable of inducing cell cycle arrest under normal cellular conditions (39-44). P14ARF tumor suppressor is a potent inhibitor of the ribosome biogenesis pathway, and is particularly activated by oncogenic stimuli (45-47). We therefore evaluated if overexpression of ARF could negate the proliferative advantage conferred by Drosha overexpression. To test this, we overexpressed Drosha and p14ARF alone or together

in TLHMECs (Figure 3.5A). Again, Drosha overexpression by itself resulted in significant gains in cell proliferation over three days (Figure 3.5B). Overexpression of p14ARF alone caused a lowering of Drosha expression (Figure 3.5A) and a dramatic decrease in cell proliferation (Figure 3.5B). Notably, overexpression of p14ARF was able to overcome the pro-proliferation effects of Drosha overexpression (Figure 3.5B), demonstrating that in absence of p53 and RB, p14ARF is able to completely restrain any positive effects of Drosha on cell proliferation.

3.5 DISCUSSION

The family of RNase III enzymes was originally ascribed roles in cleaving numerous forms of rRNAs across a number of species (48-51). These early RNase III enzymatic properties were eventually shown to also reside in human Drosha (*RNASEN* gene) (1). However, a prominent role for Drosha in miRNA processing thrust it into the spotlight and became the focus of much of the more recent functional characterization of Drosha (52,53). Indeed, the interaction of Drosha with numerous oncogenes and tumor suppressors has largely emphasized the potential role of these complexes in regulating miRNA biogenesis to drive or prevent tumorigenesis (54-56). In addition to its role in miRNA processing, it has been shown that Drosha-DGCR8 complex is capable of cleaving hairpin structures in DGCR8 mRNA in a cross-regulatory loop (28,57). However, identification of other mRNA targets of Drosha and their role in cancer is rather limited (5,58).

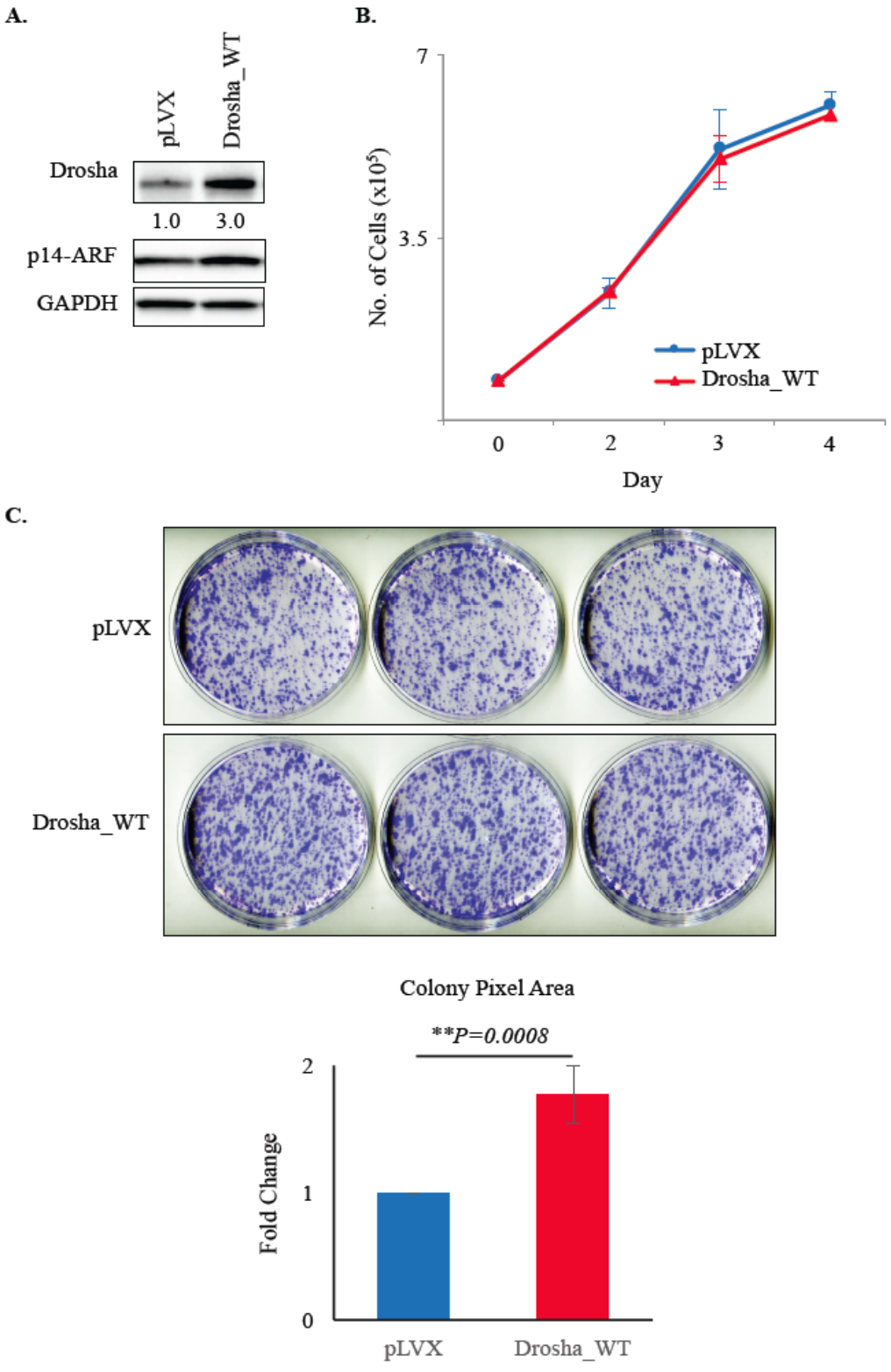
We sought to revisit the underappreciated rRNA processing role of Drosha and its effect on cell proliferation. We have shown here that not only is Drosha necessary for rRNA processing, but overexpression of Drosha is capable of enhancing rRNA biogenesis. Furthermore, we show that overexpression of Drosha is capable of increasing cell proliferation properties. Interestingly, we find that the ability of Drosha to enhance cell proliferation increases with loss of tumor suppressor functions; overexpression of Drosha in hTER immortalized primary cells only enhanced clonogenic nature of cells while Drosha overexpression in p53 and Rb impaired cells showed an increase in cell proliferation as well as clonogenic properties of cells. These results imply that Drosha's oncogenic potential may have a differential penetrance depending on the genetic background of the cells.

We have previously reported that the mouse p19ARF tumor suppressor was able to regulate Drosha mRNA translation to suppress Drosha protein expression (3), providing a possible

connection between ARF, Drosha, and nucleolar functions. Similar to the results in mouse cells, we find that the human p14ARF is capable of inhibiting Drosha protein expression (Figure 3.5A). Interestingly, we found that Drosha overexpression led to an induction of the p14ARF tumor suppressor (Figure 3.2A) in addition to an enhancement of rRNA synthesis and processing (Figure 3.4). These results once again emphasize a link between Drosha and ARF in regulation of nucleolar processes.

Additionally, we have previously shown that in mouse cells, p19ARF could function as a barrier against cellular transformation in a p53 deficient background (59). It was shown that loss of p53 led to an accumulation of p19ARF, and subsequent inhibition of p19ARF in these cells increased the tumorigenic potential of these cells when compared to p53 deficient cells by themselves. Similarly, induction of the p14ARF in the Drosha overexpressing TLHMECs could be a potential mechanism these cells employed to prevent aberrant cell proliferation, although it wasn't able to prevent Drosha from enhancing cell proliferation properties to a certain extent. This raises the question of whether Drosha may be able to induce a stronger proliferative phenotype in ARF deficient cells. Interestingly, we find that while loss of p14ARF in TLHMECs leads to an increase in Drosha protein expression, this induction was not sufficient to alter cell proliferation properties (data not shown). As forced overexpression of ARF in Drosha overexpressing cells was able to completely negate the proliferative advantage conferred by Drosha overexpression, it is possible that Drosha and ARF work in a threshold-dependent manner, where each protein is able to generate a phenotypic change in cells only when the protein levels cross a certain threshold amount. Further study of the cross-regulation between Drosha and ARF is necessary to understand the mechanisms these proteins implement in regulating cellular functions.

3.6 FIGURES


A.
pLVX
Drosha_WT
Drosha
1.0
3.0
p14-ARF
GAPDH
B.
7
3.5
No. of Cells (x10⁵)
pLVX
Drosha_WT
0
2
3
4
Day
C.
pLVX
Drosha_WT
Colony Pixel Area
**P=0.0008
2
1
0
Fold Change
pLVX
Drosha_WT

<u>Figure 3.1</u> – Drosha overexpression increases clonogenic properties of primary cells. A. Immunoblot analysis of indicated proteins in HME-hTERT cells expressing control and Flag-Drosha overexpression constructs. **B.** To analyze alterations in cell proliferation properties, 75,000 cells for each condition were plated and cells were counted on indicated days following plating. **C.** To analyze alterations in colony formation capabilities, 3000 cells for each condition were plated and colonies were stained by Giemsa stain 15 days post plating (top panel). Quantification of fold change in average pixel area covered by total number of colonies per condition.

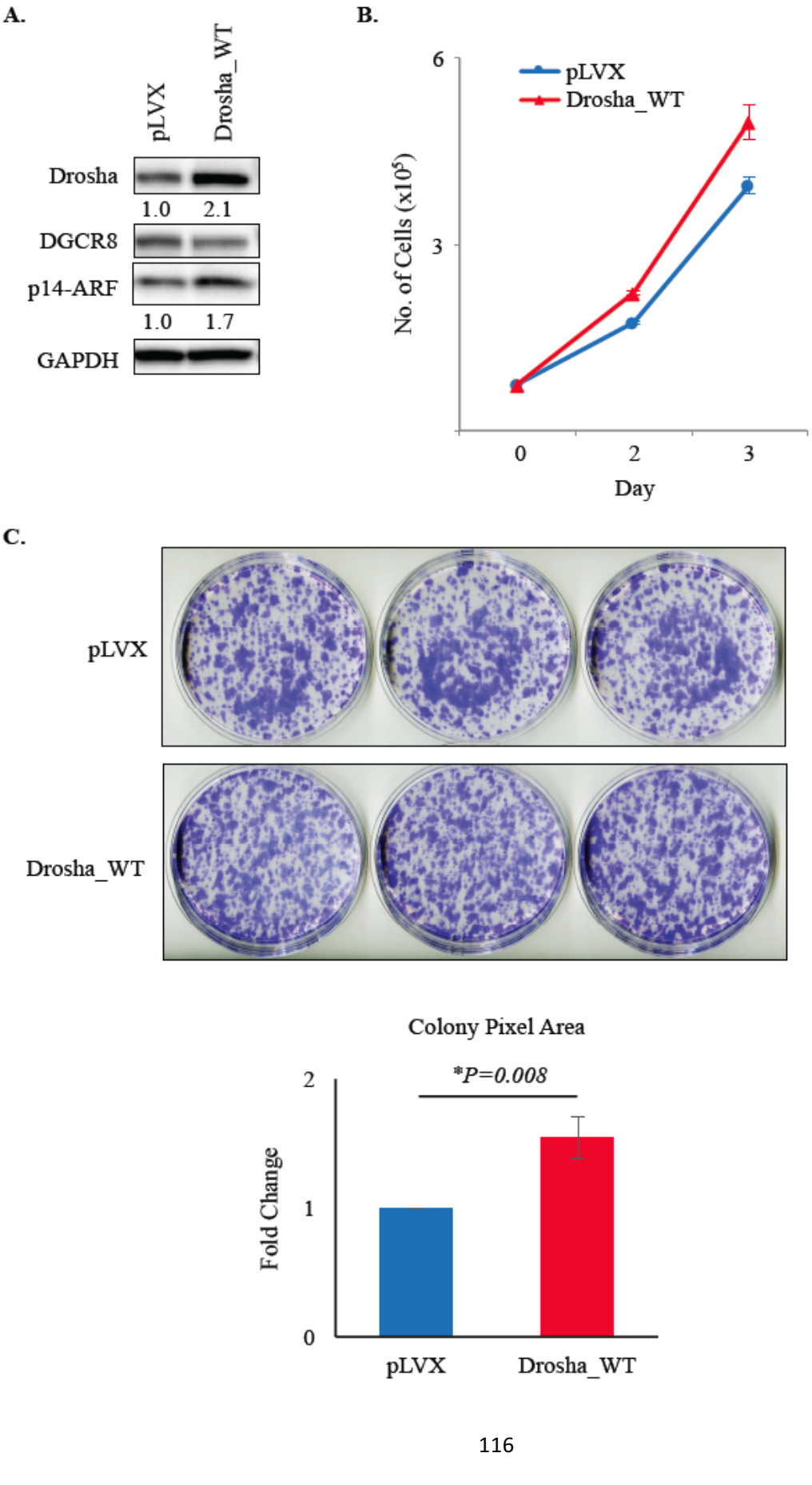
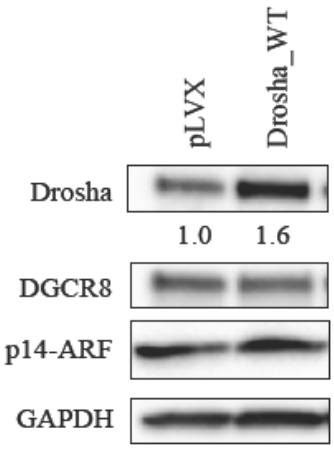

A.
pLVX
Drosha_WT
Drosha
1.0
2.1
DGCR8
p14-ARF
1.0
1.7
GAPDH
B.
pLVX
Drosha_WT
No. of Cells (x10^5)
6
3
0
2
3
Day
C.
pLVX
Drosha_WT
Colony Pixel Area
*P=0.008
Fold Change
2
1
0
pLVX
Drosha_WT

Figure 3.2 – Drosha overexpression increases cell proliferation properties of p53 and RB-impaired cells. A. Immunoblot analysis of indicated proteins in TLHMECs expressing control and Flag-Drosha overexpression constructs. **B.** To analyze alterations in cell proliferation properties, 50,000 cells for each condition were plated and cells were counted on indicated days following plating. **C.** To analyze alterations in colony formation capabilities, 3000 cells for each condition were plated and colonies were stained by Giemsa stain 15 days post plating. Quantification of fold change in average pixel area covered by total number of colonies per condition.

A.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Drosha_WT
Drosha
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1.6
DGCR8
p14-ARF
GAPDH

B.

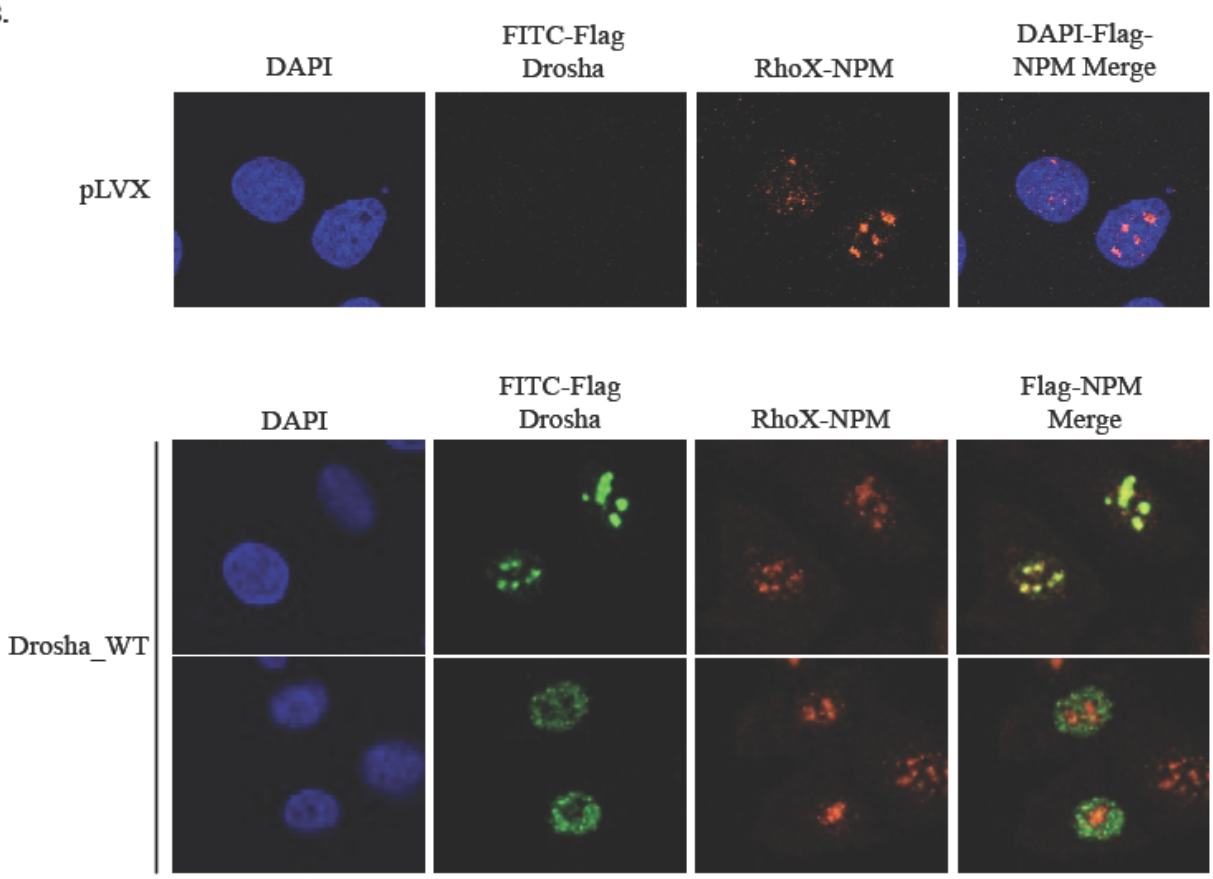
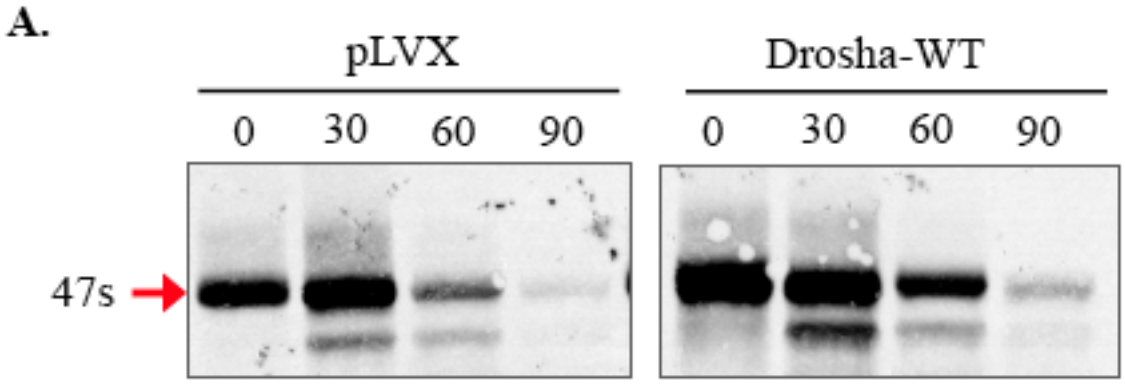

DAPI
FITC-Flag Drosha
RhoX-NPM
DAPI-Flag-NPM Merge
pLVX
DAPI
FITC-Flag Drosha
RhoX-NPM
Flag-NPM Merge
Drosha_WT

Figure 3.3 – Overexpressed Drosha protein localizes in nucleolus and nucleoplasm. A. Immunoblot analysis of TLHMECs infected with pLVX control or Flag-Drosha (WT-Drosha) was performed to confirm Drosha overexpression. **B.** Subcellular localization of Flag-Drosha was analyzed using immunofluorescence confocal microscopy on TLHEMEs infected with control or Flag-Drosha constructs. Nucleolar localization of Drosha was determined through co-localization of Flag-Drosha (green) and nucleolar marker NPM (red).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90
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30
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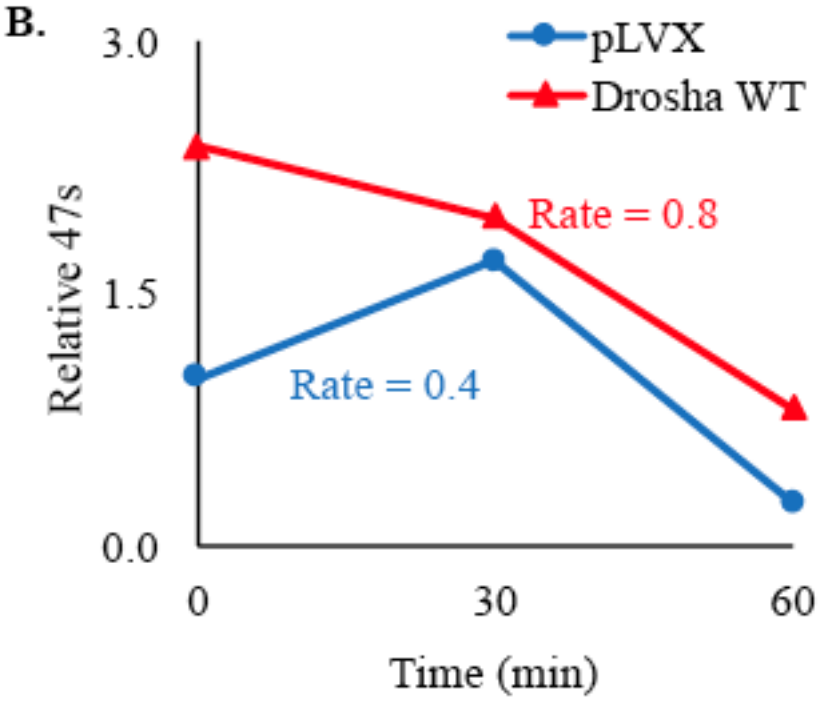
90
47s


B.
3.0
1.5
0.0
pLVX
Drosha WT
Rate = 0.8
Rate = 0.4
Relative 47s
0
30
60
Time (min)

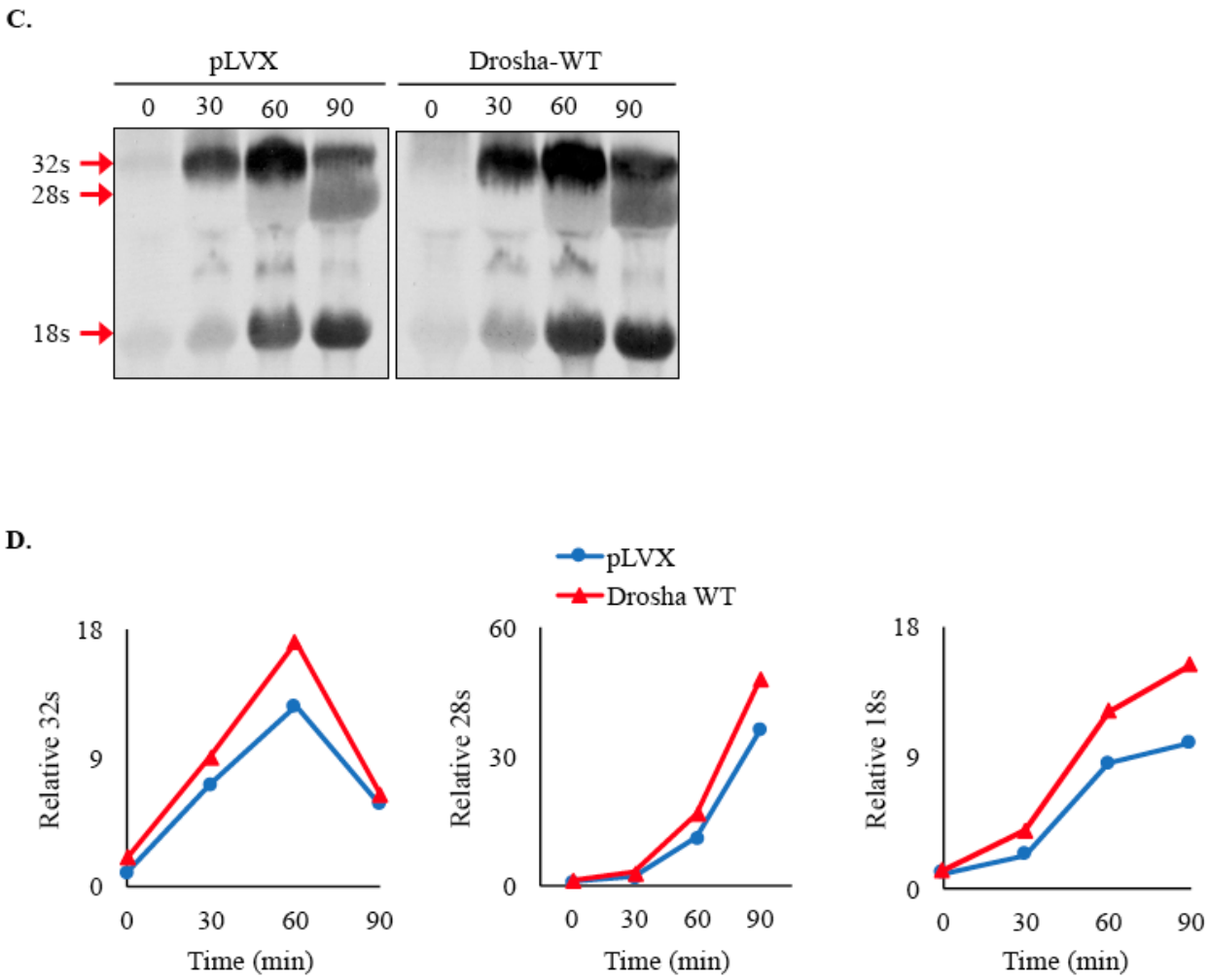
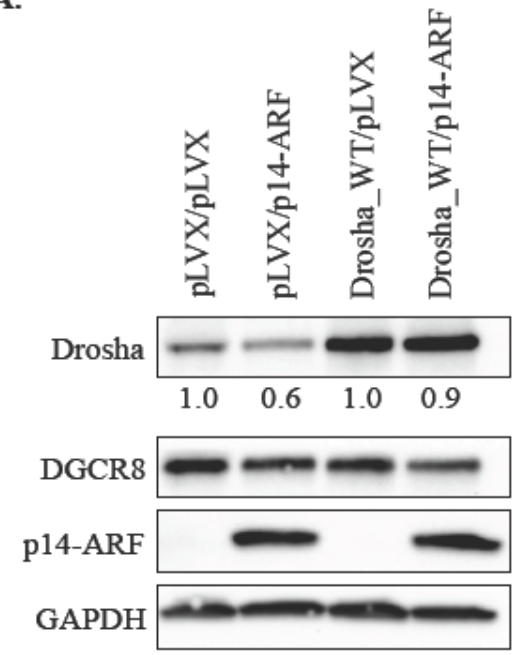


Figure 3.4 – Drosha overexpression increases rRNA processing. A, C. Control (Plvx) and Flag-Drosha overexpressing TLHMECs were labeled with [methyl-3H]-methionine and chased for indicated times. Radiolabeled RNA was separated on agarose gel, transferred to membrane and visualized by autoradiography. **B, D.** Quantification of 47s processing and 32s, 28s and 18s rRNA accumulation. Relative band intensities were obtained by individually normalizing each rRNA species to the pLVX control sample at t = 0.

A.


pLVX/pLVX
pLVX/p14-ARF
Drosha_WT/pLVX
Drosha_WT/p14-ARF
Drosha
1.0
0.6
1.0
0.9
DGCR8
p14-ARF
GAPDH

B.

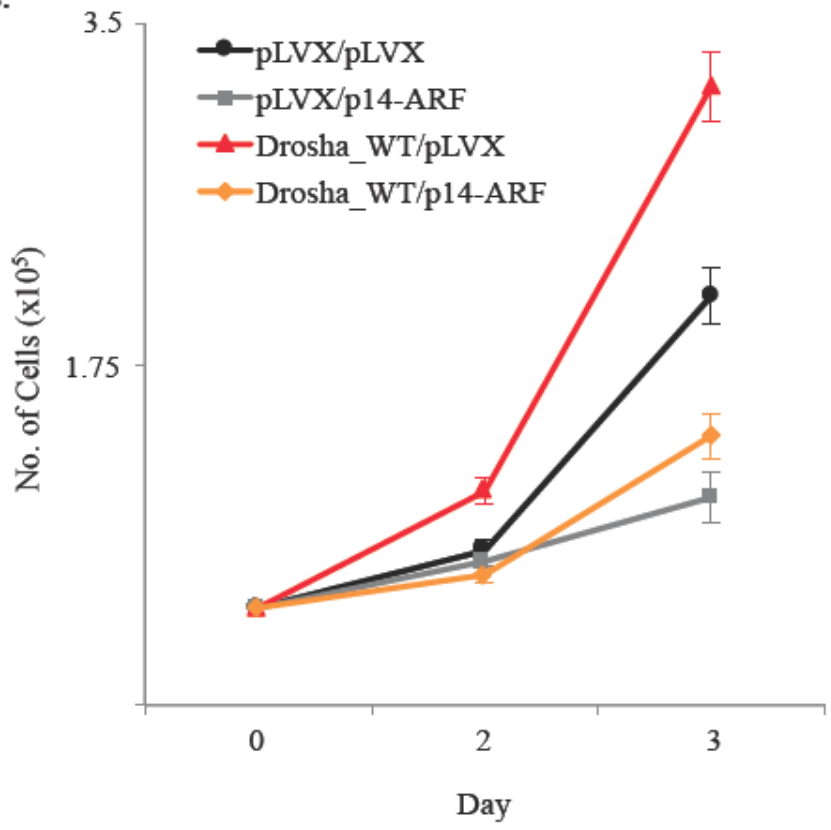

3.5
1.75
No. of Cells (x10⁵)
pLVX/pLVX
pLVX/p14-ARF
Drosha_WT/pLVX
Drosha_WT/p14-ARF
0
2
3
Day

Figure 3.5 – ARF attenuates Drosha-mediated cell proliferative advantage. A. Immunoblot analysis of indicated cell lines to confirm Drosha and ARF overexpression. **B.** To analyze alterations in cell proliferation properties, 50,000 cells for each condition were plated and cells were counted on indicated days following plating.

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CHAPTER 4:

SUMMARY AND FUTURE DIRECTIONS

4.1 SUMMARY

As the protein synthesis machinery, ribosomes play a significant role in establishing overall cell behavior. Multiple oncogenes upregulate ribosome biogenesis as a means to increase cell proliferation rates. While enhanced nucleolar functions have been noted to be a characteristic present in multiple cancers for close to a century, the role of nucleolar functions in tumorigenesis has been ignored to a large extent. However, the finding that majority of the chemotherapeutic drugs currently used for cancer treatment function through inhibition of nucleolar functions led to a new interest in the ribosome biogenesis pathway for cancer therapy. The overall objective of this dissertation work was to identify novel mechanisms involved in regulation of ribosome biogenesis. ARF tumor suppressor, a potent inhibitor of ribosome biogenesis pathway, is activated in response to several oncogenic signals and functions to suppress proliferation of aberrant cells through multiple mechanisms (1-19). Given its potent anti-proliferative functions, deletion or silencing of the CDKN2A locus occurs at high frequency in multiple cancers. Therefore, gaining a thorough understanding of ARF-dependent regulation of ribosome biogenesis in normal cellular functions and how alterations in this regulatory network can contribute to tumorigenesis will be valuable in developing therapeutic strategies for cancer treatment. We therefore focused our attention on identifying novel mechanisms by which ARF regulates ribosome biogenesis. It was recently reported that ARF regulates expression of the ribonuclease, Drosha. Drosha expression has been correlated with tumor progression and prognosis in multiple cases. As Drosha has the ability to regulate multiple RNA species within the cell, including rRNA, we chose to delineate the mechanisms by which ARF regulated Drosha expression and functions, with a particular focus on Drosha's nucleolar functions, as they have been the most understudied aspect of Drosha's cellular functions. It was reported that Drosha localized to the nucleolus in a cell cycle dependent manner

close to a decade ago. However, the mechanisms regulating Drosha's nucleolar localization and functions are yet unknown. I hypothesized that Drosha overexpression could contribute to cellular transformation through enhancement of ribosome biogenesis, and that ARF negatively regulates Drosha's nucleolar localization and functions.

I first sought to identify mechanisms by which ARF regulates Drosha expression and functions. The work presented in Chapter 2 shows that ARF negatively regulates Drosha expression in a global and localized manner; ARF loss increased overall expression levels of Drosha protein as well as nucleolar localization of Drosha. Furthermore, loss of ARF increased Drosha's association with pre-rRNA, suggesting that ARF suppresses Drosha's rRNA processing functions by inhibiting its nucleolar localization. To further address Drosha's role in the nucleolus and its contribution to cell proliferation, we overexpressed Drosha in cells and assessed if it altered nucleolar functions. The data presented in Chapter 3 shows that Drosha overexpression by itself was able to enhance ribosome biogenesis both at the rRNA transcription and rRNA processing levels. Furthermore, Drosha overexpression led to an enhanced proliferation rate in p53- and Rb-deficient cells. This enhancement occurred despite a modest induction of ARF protein expression, and overexpression of ARF in Drosha expressing cells negated the proliferative advantage conferred by Drosha overexpression. These results all point toward a feedback loop between ARF and Drosha, which can potentially contribute to cellular transformation through ribosome biogenesis pathway.

Overall, the data presented in this dissertation has identified a novel mechanism by which ARF regulates ribosome biogenesis: ARF suppresses nucleolar functions of Drosha by inhibiting its nucleolar localization. Furthermore, we found a new regulatory loop between ARF and Drosha, in which ARF negatively regulates Drosha expression, while Drosha positively regulates ARF

expression. We further found that ARF and Drosha interact with each other in high molecular weight complexes, potentially in a DNA-dependent manner. Further study of this ARF-Drosha regulatory network is necessary to understand the functional contribution of this network in cellular functions (Figure 4.1).

4.2 FUTURE DIRECTIONS

ARF-dependent regulation of Drosha expression

We have shown in Chapter 2 that loss of ARF dramatically increases Drosha protein expression; however, how this upregulation occurs is however yet to be delineated. Previously in our lab, studies in mouse cell lines indicated that ARF can negatively regulate Drosha mRNA translation, which leads to an upregulation of Drosha protein expression by translational means in ARF-deficient cells (20). Whether this mechanism of regulation is similar in human cells is not known. Additionally, our work in mouse cell lines indicates that ARF can selectively repress Drosha mRNA translation rather than globally repress all mRNA translation. Given ARF's role in suppressing ribosome biogenesis, it is possible to believe that ARF can exert a negative role in mRNA translation. However, the fact that ARF can assert selective repression over mRNA translation is suggestive of additional layers of ARF-dependent regulation over the translation machinery.

One possible mechanism for ARF's ability to regulate translation can be deduced from a previous report showing that ARF overexpression causes a decrease in polyribosome pool and an increase of 80s monosomes (21). It was further reported that the ARF overexpressing cells failed to accumulate the translational initiation factor eIF2α subunit, indicative of an impairment in translation initiation. While these results could provide a general mechanism for how ARF

regulates translation, it does not address the question of how ARF is able to selectively regulate translation of some mRNAs, like Drosha, but not others. Recently, a ribosome footprinting study done in yeast showed that, contrary to the generally held belief that 80s monosomes are translationally inactive, select mRNAs are preferentially translated by monosomes rather than polysomes (22). In fact, mRNAs containing ORFs upstream of canonical ORF seem to show a strong preference for translation by monosomes due to the longer initiation times on these mRNAs to initiate from the canonical start site. This could be a potential mechanism by which suppression of initiation factor accumulation, such as the means implemented by ARF as mentioned above, could possibly have a negative impact on mRNAs with prior handicap in translation initiation. Analysis of Drosha 5'UTR sequence revealed five ATG start sites upstream of the canonical ORF start site (Figure 4.2A). Whether relative abundance of initiation factors can alter translation of Drosha mRNA, and whether ARF's ability to negatively regulate Drosha's mRNA translation is mediated through depletion of initiation factors is yet to be tested.

In addition to the five upstream start sites before the canonical ORF, M-fold analysis of Drosha's 5'UTR sequence predicts highly stable secondary structures with ΔGs ranging between -116.90 kcal/mol to -122.90 kcal/mol (Figure 4.2B). Previous studies have indicated that translational efficiency dramatically decreases with structural complexity of 5'UTR (23). Further studies of complex 5'UTR structures revealed that RNA helicases belonging to the DEAD-box family are capable of aiding in unwinding of the secondary structures within the 5'UTR to allow for ribosome scanning for ATG start site (24). Given the number of hairpin loops predicted in Drosha's 5'UTR, it is possible that Drosha mRNA translation requires additional aid from such helicases.

Previously in our lab, mass spectroscopic analysis of alterations in nucleolar proteins in mouse embryonic fibroblasts upon ARF loss identified the DEAD-box RNA helicase DDX5 to have altered nucleolar localization in ARF-deficient cells (25). We found that while ARF had no impact on overall levels of DDX5, the nucleolar localization of DDX5 dramatically increased upon ARF loss. We have further found that upon *Arf* loss, DDX5 enhanced rRNA transcription and processing. Although this particular study focused on nucleolar aspects of ARF-dependent regulation of DDX5, another recent report suggests that ARF can inhibit DDX5's ability to bind to c-Myc and impairs c-Myc target gene transcription (26). ARF's ability to regulate DDX5 has the possibility to impact more than just transcriptional activity within the cells as DDX5 has been implicated in multiple cellular processes, including splicing, mRNA export, and nonsense-mediated mRNA decay (27-30). Furthermore, DDX3, which has been implicated to function in translational control of mRNAs with complex secondary structures in the 5'UTR (24,31) has also been shown to interact with DDX5 to regulate DDX5's nuclear localization (32). From our lab, we have shown that DDX5 associates with pre-40s and pre-60s ribosomal complexes, leading us to question if perhaps DDX5 plays a further role in regulating ribosomal functions. We hypothesized that ARF-dependent regulation of DDX5 could contribute to repression of Drosha mRNA translation. To test this hypothesis, we first assessed if DDX5 was capable of regulating Drosha expression. We found that overexpression of DDX5 increased Drosha protein expression in primary human mammary epithelial cells (Figure 4.3).

Previously, it has been reported that c-Myc is capable of transcriptionally inducing Drosha expression (33). Given DDX5's ability to act as a transcription co-factor of c-Myc, it is possible that DDX5 overexpression induces Drosha expression in a transcriptional manner via c-Myc. However, this does not rule out the possibility that DDX5 could help regulate Drosha expression

in more than just transcriptional manner. Particularly, as DDX5 has been shown to regulate c-fos expression at multiple levels including transcription, splicing and nuclear export of c-fos mRNA (30), whether DDX5 is capable of regulating Drosha expression through different means similarly, and if ARF can impact this regulation remains to be tested.

In addition to the 5'-UTR mediated translational control, the 3'-UTR has been implicated in exerting an additional level of control over mRNA translation (34). While the exact mechanisms of 3'-UTR mediated regulation of translation are not as well understood as the 5'-UTR mediated regulation, that elements in the 3'-UTR are capable of exerting activating or repressive roles on translation is irrefutable. For example, miRNA mediated silencing of mRNAs is a well-established method of 3'-UTR mediated regulation over mRNA translation. In addition to miRNA mediated regulation, poly-A tail length, binding of repressive or activating proteins in regulatory regions of 3'-UTR, and cytoplasmic poly-A element binding protein (CPEB) have all been implicated in regulating translation. Whether ARF exerts such 3'-UTR-mediated translational control over Drosha mRNA is not known.

Bioluminescence reporter assay can provide quantitative measure of alterations in gene expression between different conditions. To test which of Drosha's UTRs are necessary for ARF-dependent regulation of translation, we cloned luciferase reporter constructs with different combinations of Drosha and/or GAPDH 5'- and 3'-UTRs flanking the Luciferase gene into a pLVX-lentiviral backbone. These constructs allow us to delineate mechanisms regulating Drosha's UTR-driven translation by measuring luciferase expression (Figure 4.4). Identifying the ideal experimental conditions for using these constructs will allow us to test which of the Drosha UTRs drives luciferase expression in conditions of ARF loss as well as DDX5 overexpression. These reporter constructs can be further used to test the requirement of different initiation factors

in regulation of Drosha mRNA translation. As a previously identified target of ARF, testing the requirement of initiation factor eIF2α could be the first step in identifying the initiation factors involved in regulating Drosha mRNA translation. Alternatively, mass spectrometry analysis of proteins altered upon ARF loss, with a focus on proteins involved in translational control, can provide more putative targets to test for regulatory role in Drosha mRNA translation.

ARF-dependent regulation of Drosha's non-rRNA processing functions

Drosha's critical role in the processing of miRNAs and consequences of deregulated miRNAs in cellular transformation have been extensively studied. Addressing how Drosha overexpression upon ARF loss impacts the miRNA profile within the cells is necessary to get a full picture of how ARF loss can contribute to cellular transformation. In fact, prior studies done in our lab on mouse cells indicate that ARF loss alters expression of a subset of miRNAs (20). That ARF loss alters miRNA profiles in a selective rather than global manner, given the upregulation in Drosha upon ARF loss, hints at an additional layer of regulation within this pathway. Furthermore, it is not clear at which level of miRNA biogenesis pathway ARF exerts its selective regulation. With its ability to regulate multiple transcription factors including p53 and c-myc (16,19), it is possible that ARF loss alters miRNA expression in a transcriptional manner. Alternatively, given our observations that ARF is found in high molecular weight complexes containing Drosha and that ARF immunoprecipitates with Drosha, it is possible that ARF can modulate Drosha's microprocessor functions on a subset of miRNAs. Given prior research supporting a role for different tumor suppressors and oncogenes in complexing with and modulating the Drosha microprocessor functions (33,35,36), it is conceivable that ARF can achieve a similar means of regulation over miRNA processing. Furthermore, it has been shown

that ARF is capable of interacting with DDX5 (26), a known co-factor of the microprocessor (37) and the mediatorfor p53-dependent regulation of miRNA processing (35). Whether the ARF-DDX5 interaction is capable of feeding into the miRNA pathway remains to be tested.

In addition to its ability to process primary miRNAs, Drosha has also been implicated in directly cleaving select mRNAs (38-41). Whether Drosha overexpression upon ARF loss can have a broader impact on protein expression than those limited to the miRNA-mediated mRNA silencing remains to be tested. Furthermore, with the new found discovery of a transcriptional role for Drosha (42) and ARF's ability to function as a transcriptional repressor (16), analyzing alterations in the transcriptome upon both ARF loss and Drosha overexpression is important.

Nucleolar localization of Drosha

Early on in its discover, Drosha has been shown to be predominantly localized in the nucleus, with a fraction localizing to nucleolus during S-phase. Although it is well established that Drosha plays a role in processing pre-rRNA species, to date, no mechanism has been identified for regulation of its nucleolar localization and rRNA processing functions. In Chapter 2, we have shown in Chapter 2 that ARF loss leads to an increased localization of Drosha to nucleolus, suggesting a role for ARF in regulating Drosha's nucleolar localization. We have also shown that ARF co-immunoprecipitates with Drosha. As ARF regulates localization and functions of other nucleolar proteins by binding to them and sequestering them away from their functional zones, it would be of interest to know if ARF is capable of regulating Drosha similarly. Amino acid residues 2-14 and 82-101 of human ARF have been shown to be necessary for its nucleolar localization and many of its tumor suppressor functions (3). It has been shown that these domains are necessary for ARF's ability to bind to HDM2 and sequester it away from p53 tumor suppressor, thereby lifting

HDM2-mediated suppression of p53. We tested if these critical regions of ARF could be important for its interaction with Drosha. We found that both Δ2-14 and Δ82-101 deletion mutants of ARF continue to immunoprecipitate with Drosha similar to the wild type ARF protein (Figure 4.5). While ARF's interaction with Drosha may not require ARF's HDM2-interacting domains, it is likely that interaction between ARF and Drosha could be masking sequences necessary for Drosha's nucleolar localization. These sequences could either be nucleolar localization sequences present within Drosha protein itself, or they could belong to other nucleolar shuttling proteins that can bind to and pull Drosha into nucleolus.

Analysis of Drosha protein sequence using NoD webserver and command line program (43) predicted three potential regions between amino acids 244 and 337 to contain nucleolar localization sequences (Figure 4.6). Whether these putative residues do aid in Drosha's nucleolar localization is yet to be determined. As we found that ARF's nucleolar localization residues were not necessary for its interaction with Drosha, it would be of interest to see if Drosha's nucleolar localization signal residues are necessary for ARF interaction. Furthermore, whether ARF is capable of masking these residues to prevent nucleolar localization of Drosha needs to be tested.

Proteins can enter nucleolus either by having a nucleolar localization sequence within their protein sequence or by interacting with other nucleolar proteins. NPM, in particular, has been shown to shuttle other proteins involved in ribosome biogenesis into the nucleolus even when they lack a nucleolar localization sequence of their own. It has also been shown that ARF is capable of sequestering NPM to prevent its shuttling functions and keep proteins involved in ribosome biogenesis from entering the nucleolus. We have shown that NPM immunoprecipitates with Drosha, suggesting that NPM is capable of complexing with Drosha either directly or indirectly.

Whether this interaction between NPM and Drosha is necessary for Drosha's nucleolar localization remains to be tested.

Nucleolar functions of Drosha

Chapter 2 of this Dissertation identified ARF as a regulator of Drosha's nucleolar localization. However, the steps in ribosome biogenesis pathway that are impacted by ARF-dependent regulation of Drosha are not known. Prior reports have indicated that inhibition of Drosha functions can lead to an accumulation of 32s and 12s rRNA precursors (44), suggesting a role for Drosha in processing these precursors. As demonstrated in Chapter 2, ARF loss increases precipitation of select bands (potentially 32s and 28s) with Drosha antibody when compared to ARF proficient cells, suggesting ARF can inhibit Drosha's association with select rRNA precursors. Additionally, the 18s rRNA immunoprecipitated with Drosha as well, although there was no noticeable difference in the amount of precipitated 18s between TLHMECs and CR-p14ARF cells. Association of Drosha with the mature 28s and 18s rRNAs may be indicative of a more involved role for Drosha in ribosome biogenesis than merely as an RNase enzyme necessary for processing of rRNA precursors. Whether Drosha continues to associate with the ribosomal subunits as they get assembled and shuttled out to the cytoplasm, and how this association may impact mRNA translation remain to be tested.

To further test Drosha's nucleolar roles, we overexpressed Drosha and analyzed the consequences on the nucleolar compartment. In Chapter 3, we showed that overexpression of Drosha enhanced 47s synthesis as well as the rRNA processing rate when compared to control cells. We further showed that Drosha overexpressing cells have an increased cell proliferation rates when compared to control cells. Given that Drosha participates in processing of multiple species

of RNAs, determining the contribution of each of these arms in maintaining cellular homeostasis versus promoting cellular transformation can be tricky. Furthermore, alterations in Drosha have been suggested to have tumor suppressive role in some cancers while promoting tumorigenesis in others (45-63). This dual nature of Drosha in tumorigenesis could possibly depend on which arm of its functions are altered in given cancer. It is therefore imperative to determine the contribution of each arm of Drosha's functions in cellular transformation, so as to identify weak links that can be exploited for therapy. While the miRNA functions of Drosha have been extensively studied, Drosha's role in rRNA processing and mRNA processing have so far taken a back stage. Since ribosome biogenesis is most often deregulated in cancer cells and that inhibitors of this pathway are currently being tested for cancer therapy, determining the extent to which Drosha participates in this pathway and how its participation is regulated can identify new targets for therapy. Data presented in Chapter 3 of this Dissertation indicates that Drosha overexpression is capable of enhancing cellular proliferation. However, as our construct was overexpressed in both the nucleoplasmic and nucleolar compartments, it is difficult to determine if both the miRNA-processing and rRNA processing arms of Drosha are necessary for this increased proliferation properties, or if either one of these arms is sufficient for this enhancement. To identify the contribution of Drosha's nucleolar functions to cellular transformation, cloning a Drosha ORF construct tagged with a nucleolar targeting sequence previously identified in known nucleolar proteins (3,64,65) (Table 4.1) would allow us to selectively overexpress nucleolar Drosha. Utilizing such construct will allow us to test whether upregulation of nucleolar functions of Drosha without altering its miRNA processing arm will have an impact on cell proliferation properties.

In addition to determining the role of Drosha's nucleolar functions in cellular transformation, identifying mechanisms regulating these functions is necessary for determining

appropriate therapeutic targets. Data presented in Chapter 2 identifies ARF as a possible regulator of Drosha's nucleolar localization. Furthermore, immunoprecipitation of Drosha complexes from whole cell protein lysates revealed that ARF precipitates with Drosha in RNA-independent manner. However, ARF fails to precipitate with high molecular weight protein complexes containing Drosha in presence of DNase and RNase, suggesting a potential DNA-dependent interaction between the two proteins. As Drosha has been shown to participate in Pol II-dependent mRNA transcription (42) and ARF has the ability to function as a transcriptional repressor (16,66), it is worthwhile to assess if the apparent DNA-dependent interaction between the proteins occurs as part of a transcriptional complex, and in particular if this complex can form over rDNA promoter. Although a role for Drosha in ribosome biogenesis has been limited to rRNA processing, we found that Drosha overexpression in p53 and Rb deficient cells was able to increase 47s rRNA transcript levels, suggesting that Drosha could function in rRNA transcription in addition to rRNA processing.

In the case of rRNA processing, whether Drosha is capable of acting independently in its rRNA processing functions, or if it requires other co-factors to perform its functions is not known. It has long since been established that Drosha requires DGCR8 for substrate specificity in pri-miRNA processing (67). However, little is known regarding co-factors required for Drosha's proper functioning in rRNA processing. We have shown in Chapter 2 that, like Drosha, DGCR8 localizes to both nucleoplasm and nucleoli of different human mammary cell types. Other reports have also indicated that DGCR8 is capable of localizing to the nucleolus in a transcription-dependent manner (68). Recently, it has been demonstrated that ARF is capable of regulating DGCR8 by means of sumoylation and that this ARF-mediated sumoylation of DGCR8 has tumor suppressive role (69). Given DGCR8's nucleolar localization, whether it aids Drosha in rRNA

processing remains to be tested. Although the Drosha-interacting domain in DGCR8 has been identified (70), disrupting interaction between Drosha and DGCR8 could impact miRNA pathway in addition to rRNA pathway, making it difficult to determine the direct role these proteins play in rRNA processing. Therefore, rather than directly disrupting Drosha and DGCR8 interaction, finding means to prevent DGCR8's nucleolar localization could be a way to determine if DGCR8 is necessary for Drosha's ability to process rRNA. It has been shown previously that C-terminal region of DGCR8 is necessary for interaction with Nucleolin (Figure 4.7A), a protein that plays an important role in nucleolar functions as well as in DGCR8's nucleolar localization (68). NoD webserver command line program predicted a single region between residues 657 and 676 of DGCR8 to contain a putative nucleolar localization signal (Figure 4.7B). Identifying a point mutation in this region that could inhibit nucleolar localization without altering Drosha-binding would be ideal to test for nucleolar requirement of DGCR8 in rRNA processing. We would presume that unhindered binding of the mutant DGCR8 to Drosha would leave the miRNA processing arm unaltered, and hence the functional consequences of preventing nucleolar localization of DGCR8 can be assessed with this mutant. On the other hand, many proteins with nucleolar functions can localize to the nucleolus even if they lack a nucleolar localization signal by piggy-backing on other nucleolar proteins. If DGCR8 is capable of entering nucleolus in such manner, mutations within its nucleolar localization signal may not have a huge impact on its nucleolar localization. Particularly, if DGCR8 uses Drosha to localize to the nucleolus, making mutations within the DGCR8-Drosha binding region may be necessary to assess DGCR8's necessity in rRNA processing.

In addition to DGCR8, DDX5 has been shown to associate with Drosha microprocessor and aid in processing of a subset of miRNAs (37). Previous publications from our lab as well as

data presented in Chapter 2 of this Dissertation indicate that DDX5 has critical nucleolar functions and is capable of complexing with Drosha. Further characterizing the nucleolar interaction between Drosha and DDX5 could identify additional regulatory mechanisms involved in Drosha's nucleolar functions. Moreover, Drosha overexpression in the TLHMECs enhanced 47s transcription in addition to enhanced rRNA processing. As Drosha has been shown to bind Pol II and transcriptionally regulate select target genes, it would be of great interest to assess if Drosha is also capable of binding Pol I and regulate rDNA transcription.

4.3 FIGURES

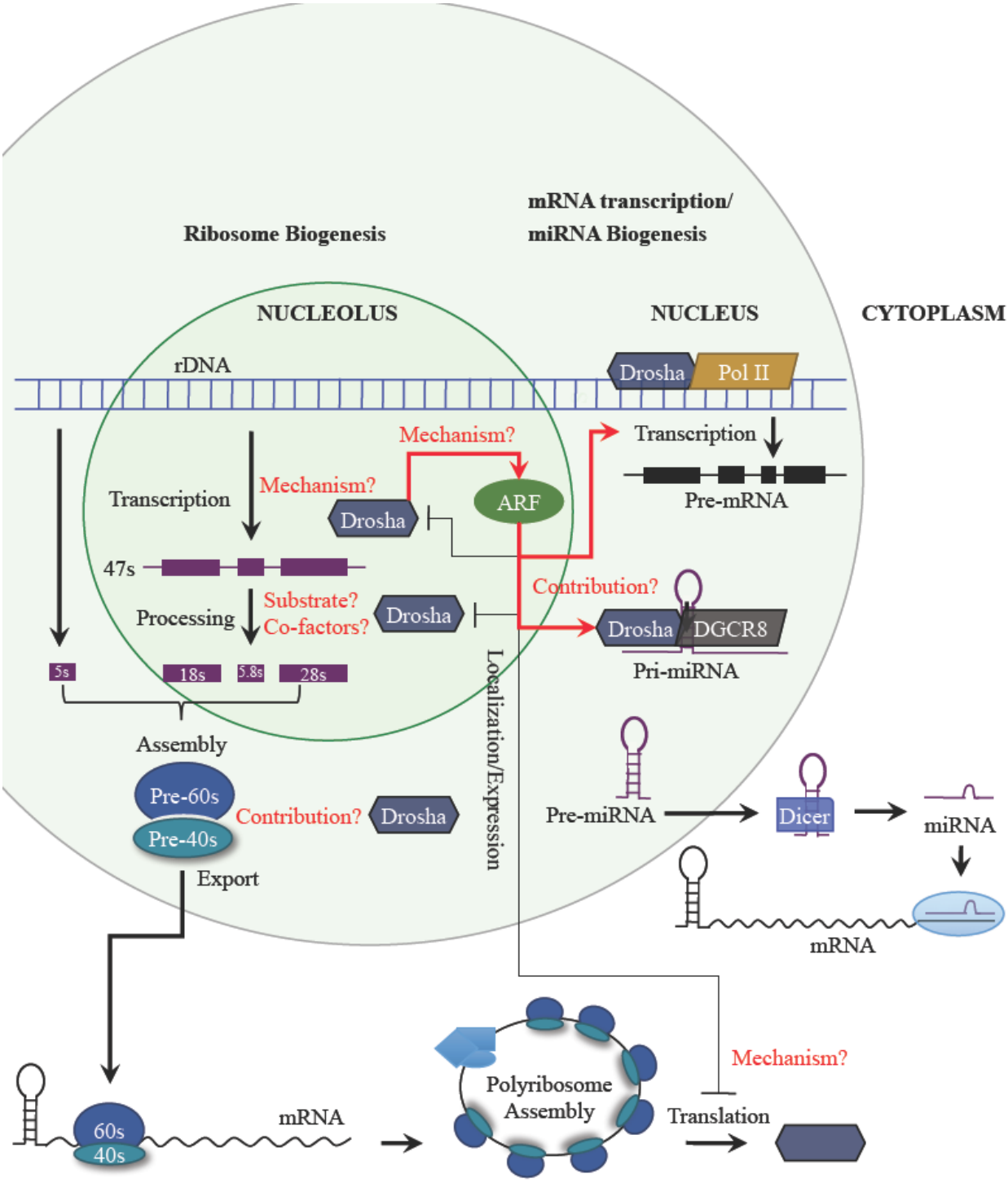
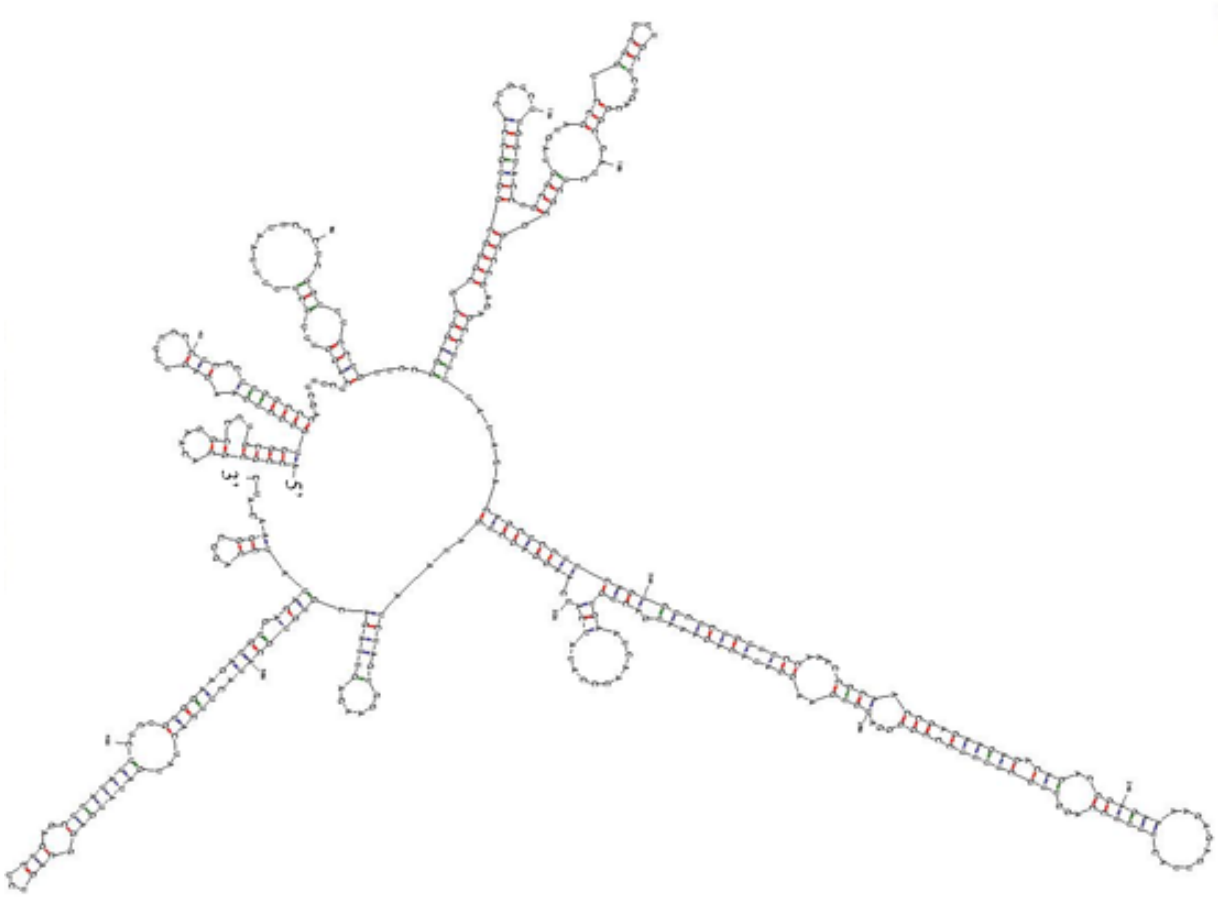

Ribosome Biogenesis
mRNA transcription/
miRNA Biogenesis
NUCLEOLUS
NUCLEUS
CYTOPLASM
rDNA
Drosha
Pol II
Transcription
Pre-mRNA
Mechanism?
Transcription
Mechanism?
Drosha
ARF
47s
Contribution?
Processing
Substrate?
Co-factors?
Drosha
Drosha
DGCR8
Pri-miRNA
5s
18s
5.8s
28s
Assembly
Localization/Expression
Pre-60s
Pre-40s
Contribution?
Drosha
Pre-miRNA
Dicer
miRNA
Export
mRNA
60s
40s
mRNA
Polyribosome
Assembly
Mechanism?
Translation

Figure 4.1 – Summary and Future Directions. ARF-mediated regulation of Drosha's functions are summarized. Additionally, future avenues of research are indicated in red font. In brief, ARF regulates nucleolar localization of Drosha and thereby inhibits its rRNA processing functions. The exact pre-rRNA substrate processed by Drosha needs to be determined, along with the cofactors, if any, necessary for cleaving of rRNA substrates by Drosha. We also see an increase in 47s pre-rRNA transcription upon Drosha overexpression, although we do not know yet if Drosha is capable of directly effecting 47s transcription. Induction of ARF upon Drosha overexpression further points toward a negative feed-back loop and needs to be further characterized. ARF-dependent regulation of Drosha mRNA translation has been proposed in mouse cells and needs to be tested in human cells, along with the mechanism by which ARF regulates translation of select group of mRNAs. Give Drosha's role in miRNA biogenesis, mRNA cleavage and mRNA transcription, the impact ARF loss has on all of these processes needs to be assessed, particularly given its inhibitory role in regulating Drosha's expression.

A. Drosha 5'UTR Sequence

5' – A CCGCGCACAAGGCCCTGCGGTGGGCTGAAGAGTTTTCCCTCCCTTGGCCCAGC
TTTCTCAGGTTTGCTTTTTAATTCCCTCGGTTTCCTGTTCCGGAGGCGCGGGCGGTGC
CACTGTCTTGGTACCTGCGGTAGTAGCCTGGCTTTGCTCTGACGGCGATCTCGCGGC
CCGAGAGCCTTTTATAGACAGCTCCTTCAGTGTCTCTGTTTCCAAACCGCAACCGAG
AAGAGACAGACGGAGAAAAGAGAGTTACTTTTCCAGGTTGCTTTTCCCGGGGATGT
GAAGGATACAGAAATGACTGTGAATCAACCCATATCATCAAGGAGCTGATAATCTA
GTGGAAGAGTTAGACGTGTGCATACTTCACTATGATATGAGGCAGTCTCTGAGCTTA
TATTCTCTGTGGAAGATGTGACATATCCAGGCGGAACATC – 3'

B. Drosha 5' UTR M-fold Predicted Structure



C. Drosha 3'UTR Sequence

5' – AGGAGGGCATGCAAGTGTGGAGTATTTACTTGCTCAGTAACTGTGACTGTTGT
CTATTGAGACCTAGCCTAGTTTTCCTGCAGACAATGAATGAAGTGTGCTCATTGAAA
TAAAATACAGAGTCAAATCGCTATTGTTGTTTTAATGATCTGTTTTTAGCTGGATGGT
CTTTATTACAAAGTATTAGATTTTTCTTCTATTTAACGGAAAACTTGACTTTGGTGAA
TGTGCATTACTTCCTTTTATTTTGCTCTTTAAATAATAAAATTCAAGAAGCATATTCT
ATGTGGAATAGATCCTGTTTTTCCATCTGTGTCCCAGATTGTGACCCTAGACTTTCAA
TTGACAAGTAAAAAATTGACTTTACTAGACATTTTGACTGTGCTCTAGTAACATCTA
TCCTTTTTCAAATCTCTGGATTTTTAAGTAGATTGTTCAGCTTTCATCCGGTGGCTGTT
CATCAAGTTATCAGCTGCAAATATTGAACTTACCTCTCTCTAAGCAGTGAGTGTTTTG
TAGAAGGAATCCGTTTAACAATTAATTGGCTAATGGGAGAAGGGGAAAGACTGATA
TTCAAGTCATACAGATTCTTTGAATCATTAGAATAGGAGAGAAATCATGATTCTAAG
CCAGGCCACACTTTAAACCAAGTGCTCTCACCCTGGGGTTAGTGGAACCTTTAAGAA
GTTAATGAACAGACTTCAAGGAAGTCAAAAACCTCCCAATACTATATTCAATTTTCT
GTGTGTGTTTGAGATTTGAGAGGGCATGGGGAGCAGGAAGGAGGAGGGTTTATAGC
TTTTATCAGCCTCTCTAAGTGGGCCCTGCAGTAAAAGGCTAACATGACATTCAAAGA
CATAACATTTTAAAAAAAGTTATTCCAAACTAAACATCACTGGTTTCTTATTAATAA
AGGCAAAACTTCTTTGTAAAACAAAAA– 3'

D. Drosha 3' UTR M-fold Predicted Structure

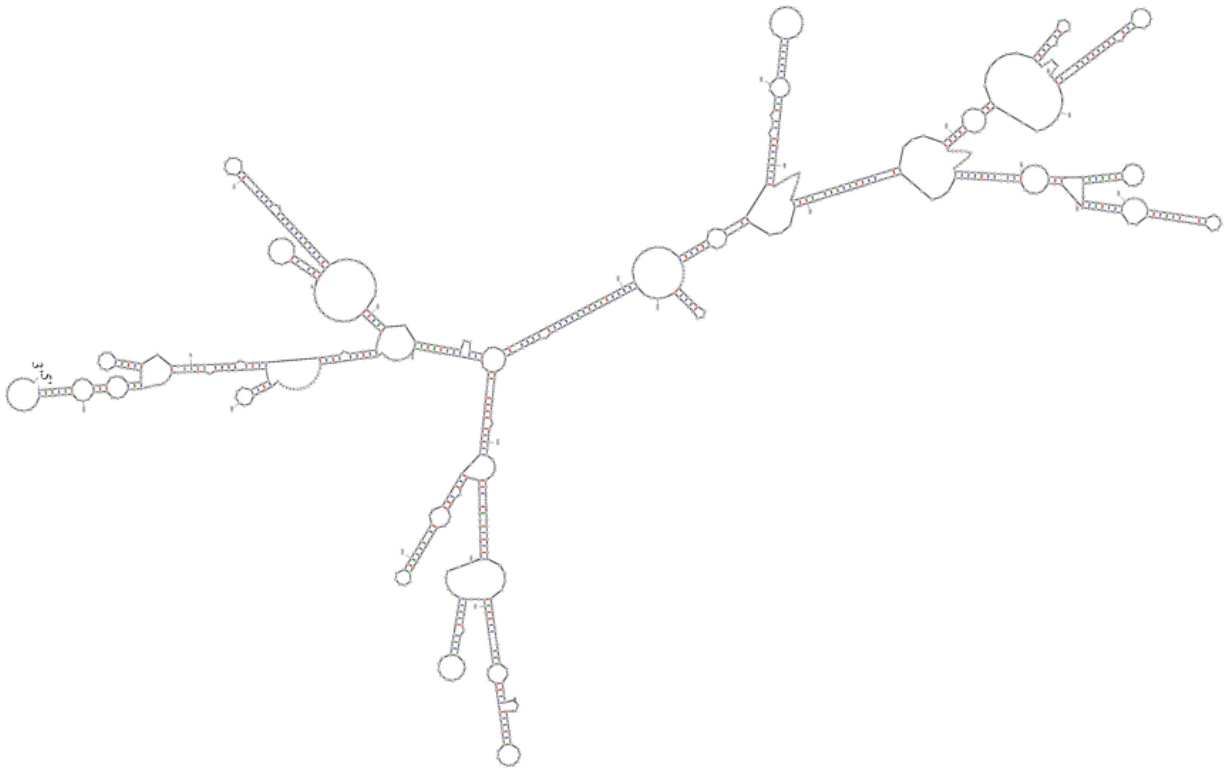


Figure 4.2 – Primary sequence and secondary structure of Drosha 5'- and 3'-UTRs. cDNA was generated from RNA isolated from TLHMECs. The 5'- and 3'- UTRs of Drosha were obtained through Rapid amplification of cDNA ends (RACE) (**A,C**). The secondary structures of the acquired sequences were predicted using the M-fold RNA folding application (**B,D**).

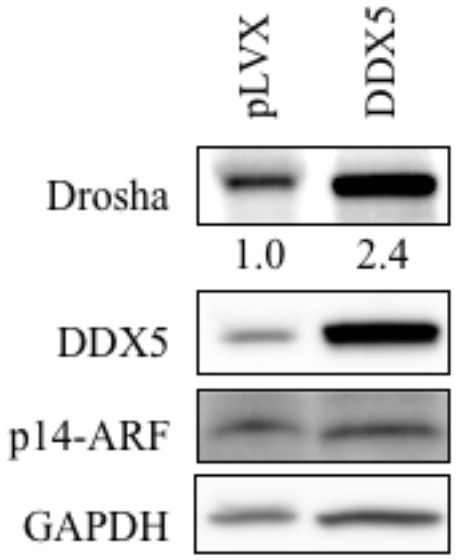


Figure 4.3 – DDX5 positively regulates Drosha expression. DDX5 was overexpressed in primary human mammary epithelial cells (HMECs) and alterations in indicated proteins was measured using immunoblotting.

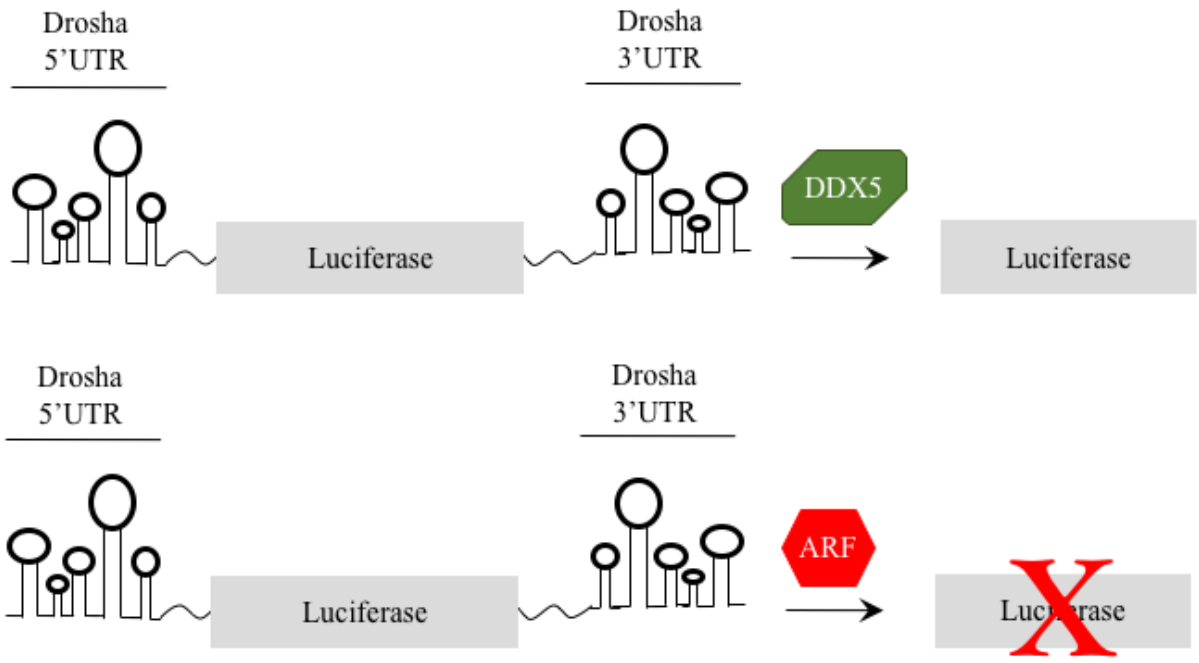


Figure 4.4 – Bioluminescence Luciferase Reporter Assay. Schematic representation of luciferase reporter construct flanked by Drosha 5'- and 3'-UTRs and predicted outcomes of reporter expression in presence of DDX5 and ARF.

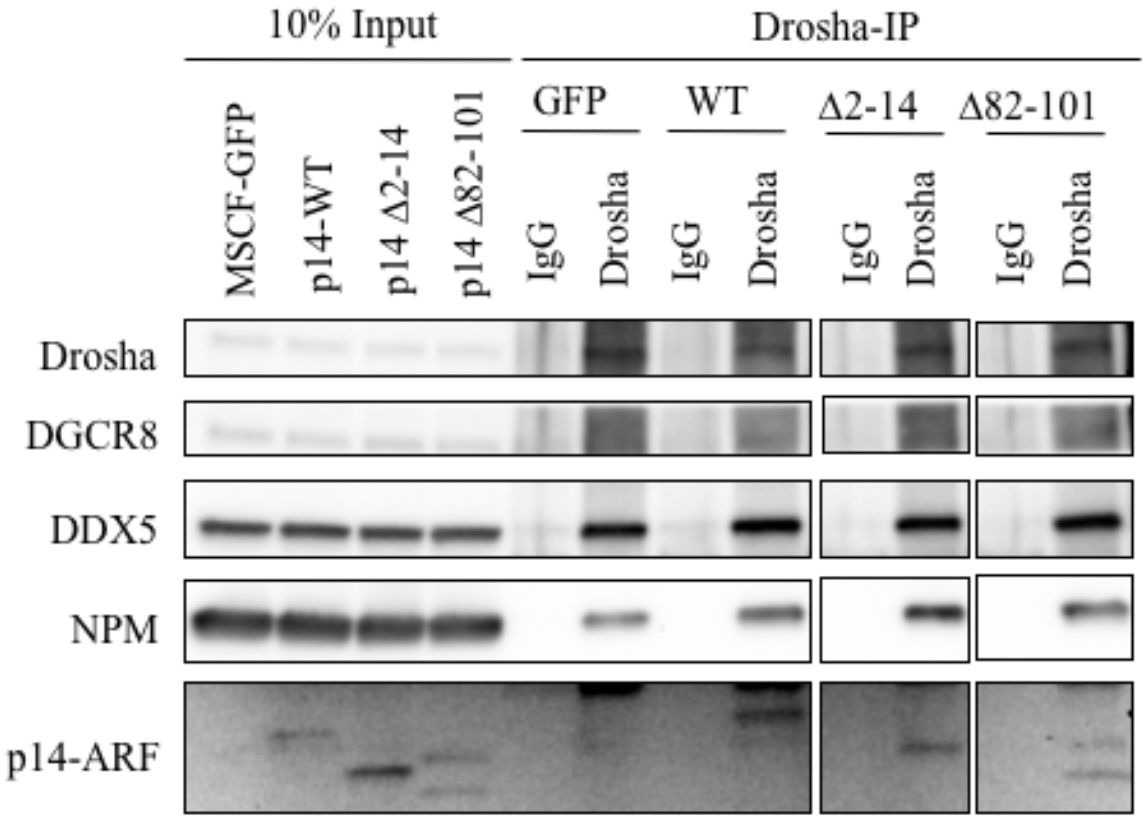


Figure 4.5 – ARF immunoprecipitates with Drosha. Wild type ARF and deletion mutants (D2-14 and D82-101) lacking nucleolar localization signal in ARF were overexpressed in human mammary epithelial cell line, MCF10A. Drosha-complexes were immunoprecipitated using Drosha-specific antibody and immunoblot analysis was performed for indicated proteins.

http://www.compbio.dundee.ac.uk/nod
Michelle S. Scott, Peter V. Troshin and Geoffrey J. Barton "NoD: a Nucleolar localization sequence detector for eukaryotic and viral proteins" - *BMC Bioinformatics* 2011, 12:317, doi:10.1186/1471-2105-12-317.

Query protein is RNASEN (NP_001093882)

3 NoLSs are predicted in this protein:

HRSLDRRERGRSPDRRRQDSRYRSDYD (between positions 244 and 270)

HRSYERSRERERERHRHRDNRRSPSLER (between positions 278 and 305)

KRSGSRSPSREKKRARWEEEKDRWSD (between positions 312 and 337)

Position in full-length protein (NoLSs shown in red):

MMQGNTCHRMSFHPGRGCPRGRGGHGARPSAPSFRPQNLRLLHPQQPPVQYQYEPPSAPSTTFSNSPAPNFLPPRPD
FVPFPPPMPPSAQGPLPPCPIRPPFPNHQMRHPFPVPPCFPPMPPPMPCPNNPPVPGAPPGQGTFPFMMPPPSMPHPPPP
PVMPQQVNYQYPPGYSHHNFPPPSFNSFQNNPSSFLPSANNSSSPHFRHLPPYPLPKAPSERRSPERLKHYDDHRHRD
HSHGRGERHRSLDRRERGRSPDRRRQDSRYRSDYDRGRTPSRHRSYERSRERERERHRHRDNRRSPSLERSYKKEY
KRSGSRSPSREKKRARWEEEKDRWSDNQSSGKDKNYTSIKEKEPEETMPDKNEEEEEELLKPVWIRCTHSENYYSS
DPMDQVGDSTVVGTSRLRDLYDKFEEELGSRQEKAKAARPPWEPPKTKLDEDLESSSESECESDEDSTCSSSSDSEV
FDVIAEIKRKKAHPDRLHDELWYNDPGQMNDGPLCKCSAKARRTGIRHSIYPGEEAIKPCRPMTNNAGRLFHYRITV
SPPTNFLTDRPTVIEYDDHEYIFEGFSMFAHAPLTNIPLCKVIRFNIDYTIHFIEEMMPENFCVKGLELFSLFLFRDILEL
YDWNLKGPLFEDSPPCCPRFHFMPRFVRFLPDGGKEVLSMHQILLYLLRCSKALVPEEEIANMLQWEELEWQKYAE
ECKGMIVTNPGTKPSSVRIDQLDREQFNPDVITFPIIVHFGIRPAQLSYAGDPQYQKLWKSYVKLRHLLANSPKVKQT
DKQKLAQREEALQKIRQKNTMRREVTVELSSQGFWKTGIRSDVCQHAMMLPVLTHHIRYHQCLMHLDKLIGYTFQ
DRCLLQLAMTHPSHHLNFGMNPDHARNSLSNCGIRQPKYGDRKVHHMHMRKKGINTLINIMSRLGQDDPTPSRINH
NERLEFLGDAVVEFLTSVHLYYLFPSLEEGGLATYRTAIVQNQHLAMLAKKLELDRFMLYAHGPDLCRESDLRHA
MANCFEALIGAVYLEGSLEEAKQLFGRLLFNDPDLREVWLNYPLHPLQLQEPNTDRQLIETSPVLQKLTEFEEAIGVI
FTHVRLLARAFTLRTVGFNHLTLGHNQRMEFLGDSIMQLVATEYLFIHFPDHHEGHLTLLRSSLVNNRTQAKVAEE
LGMQEYAITNDKTKRPVALRTKTLADLLESFIAALYIDKDLEYVHTFMNVCFFPRLKEFILNQDWNDPKSQLQQCC
LTLRTEGKEPDIPLYKTLQTVGPSHARTYTVAVYFKGERIGCGKGPSIQQAEMGAAMDALEKYNFPQMAHQKRFIE
RKYRQELKEMRWEREHQEREPDETEDIKK

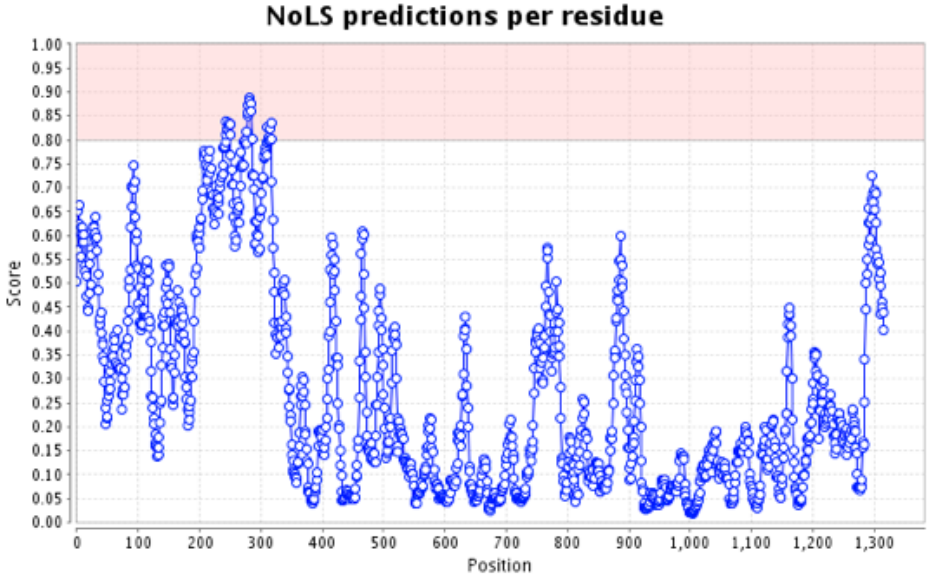
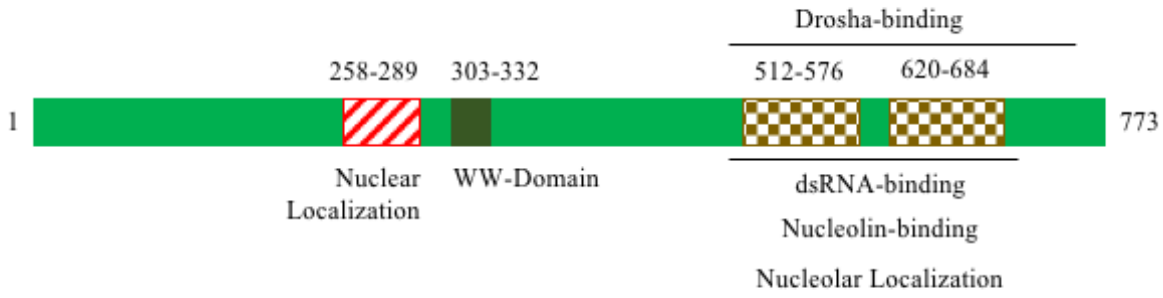


Figure 4.6 – Nucleolar localization sequence prediction in Drosha protein sequence. Drosha protein sequence was analyzed for nucleolar localization signal sequences using the NoD Nucleolar localization sequence detection program. The predicted nucleolar localization sequences are highlighted in red, with the most likely RRER motif commonly found in nucleolar localization signals underlined. The graph depicts the sequence segments that meet the nucleolar localization sequence cutoff scores of 0.8 and above.

Table 4.1. Nucleolar localization sequences of various proteins.

Protein	Nucleolar Localization Sequence
ApLLP, Aplysia LAPS18-like protein	MAKSIRSKHRRQMRMMKRE
FGF2, fibroblast growth factor 2	RSRKYTSWYVALKR
GGNNVα, betanodavirus greasy grouper nervous necrosis virus protein α	RRRANNRRR
HIC p40, human I-mfa domain-containing protein, p40	GRCRRLANFGPRKRRRRRR
HIV-1 Rev, human immunodeficiency virus-1 regulator of virion protein	RRNRRRRWRERQRQI RKKRRQRRRAHQ
HIV-1 TAT, human immunodeficiency virus-1 transactivator of transcription protein	RKKRRQRRRAHQ RQARRNRRRRWRER
HSV type 1 γ(1) 34.5, herpes simplex virus type 1 γ(1) 34.5 protein	MARRRRHRGPRRPRPP
IBV N, infectious bronchitis virus nucleocapsid	WRRQARFK
LIMK2, LIM kinases 2	KKRTLRKNDRKKR
MDM2, murine double minute 2 protein	KKLKKRNK
MDV, Marek disease virus	RRRKRNRDARRRRRKQ
NIK, nuclear factor-κB inducing kinase	RKKRKKK
PRRSV N, porcine reproductive and respiratory syndrome virus nucleocapsid	PGKKNKKKNPEKPHFP LATEDDVRHHFTPSER
Nuclear VCP-like protein	KRKGKLKNKGSKRKK
Histone H2B	KAQKKGGKKRKRSRK
HDM2	KKLKKRNK
L5	VYEKKPKREVKKKR
S6	RIALKKQRTKKNK
HTLV-1 rex	KTRRRPRRSQRSQRK
Nucleophosmin	VPQKKVK

A. DGCR8 Protein Domains



B. DGCR8 Nucleolar Localization Sequence Prediction

One NoLS is predicted in this protein:

CGKHTVRGWCKNKRVGKQLA (between positions 657 and 676)

Position in full-length protein (NoLSs shown in red):

METDESPSPLPCGPAGEAVMESRARPFQALPREQSPPPPLQTSSGAEVMDVGSGGDGQSELPAEDP
FNFYGASLLSKGSFSKGRLLIDPNCSGHSPRTARHAPAVRKFSPDLKLLKDVKISVSFTESCRSKDR
KVLYTGAERDVRAECGLLLSPVSGDVHACPFGGSVGDGVGIGGESADKKDEENELDQEKRVEYA
VLDELEDFTDNLELDEEGAGGFTAKAIVQRDRVDEEALNFPYEDDFDNDVDALLEEGLCAPKKRR
TEEKYGGDSDHPSDGETSVQPMMTKIKTVLKSRGRPPTEPLPDGWIMTFHNSGVPVYLHRESRVV
TWSRPYFLGTGSIRKHDPPLSSIPCLHYKKMKDNEEREQSSDLTPSGDVSPVKPLSRSAELEFPLDE
PDSMGADPGPPDEKDPLGAEAAPGALGQVKAKVEVCKDESVDLEEFRSYLEKRFDFEQVTVKKF
RTWAERRQFNREMKRKQAESERPILPANQKLITLSVQDAPTKKEFVINPNGKSEVCILHEYMQRVL
KVRPVYNFFECENPSEPFGASVTIDGVTYGSGTASSKKLAKNKAARATLEILIPDFVKQTSEEKPKD
SEELEYFNHISIEDSRVYELTSKAGLLSPYQILHECLKRNHGMGDTSIKFEVVPGKNQKSEYVMAC
GKHTVRGWCKNKRVGKQLASQKILQLLHPHVKNWGSLLRMYGRESSKMVKQETSDKSVIELQQ
YAKKNKPNLHILSKLQEEMKRLAEEREETRKKPKMSIVASAQPGGEPLCTVDV

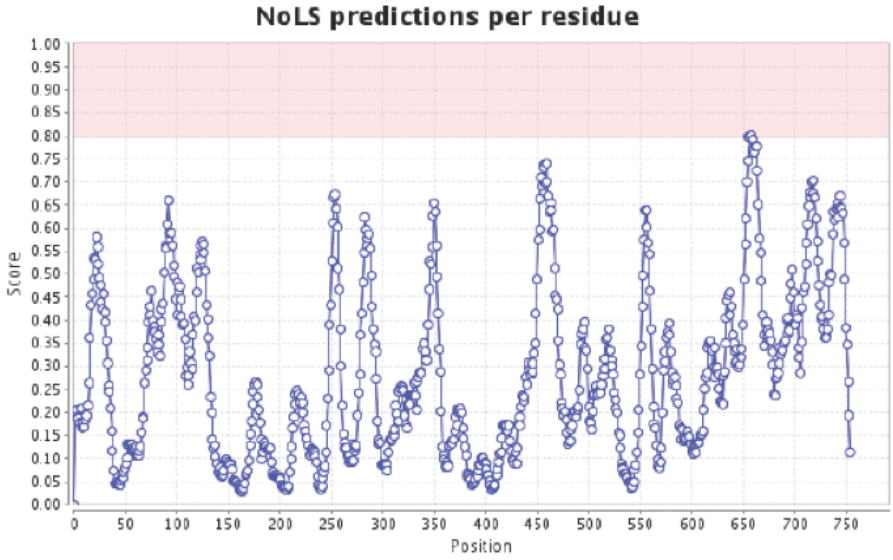


Figure 4.7 – DGCR8 domains. A. Schematic representation of DGCR8 protein domains. Functional regions such as the WW-domain, the double stranded RNA binding domain (dsRNA) and Drosha-binding domain are indicated. Additionally, regions necessary for both nuclear and nucleolar localization have also been indicated. **B.** DGCR8 protein sequence was analyzed for nucleolar localization signal sequences using the NoD Nucleolar localization sequence detection program. The predicted nucleolar localization sequence is highlighted in red. The graph depicts the sequence segments that meet the nucleolar localization sequence cutoff scores of 0.8 and above.

4.4 REFERENCES

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